Speciation and secondary contact in a colourful agamid, *Ctenophorus decresii*



Caroline Mei-Xu Dong

ORCID identifier: 0000-0003-3352-4006

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Speciation and secondary contact in a colourful agamid, *Ctenophorus decresii*

by Caroline M. Dong © 2019

Supervisors: Devi Stuart-Fox, Adnan Moussalli, Claire McLean

Title page: *Ctenophorus decresii* hybrid male (photo: Charlotte Nitschke)

Dedicated to my parents, Yuqin Sun and Jian Dong.

Abstract

Colour polymorphism, the co-existence of multiple heritable colour morphs within an interbreeding population, is thought to promote rapid phenotypic evolution and speciation. This is based on the importance of colour signals in reproductive isolation in combination with the underlying genetic architecture of polymorphism, where morphs are predicted to be governed by few genes of major effect. This prediction is supported by empirical data and stems from how colour morphs often differ in suites of co-adapted traits. During secondary contact between populations that differ in morphs, there is expected to be a high probability of genetic incompatibilities between morphs due to a breakdown of adaptive genetic correlations. Furthermore, colour signal divergence may also be accompanied by changes in behaviour and/or mating preferences leading to incompatibilities between populations which differ in morphs. These factors together may facilitate the formation of reproductive isolation and ultimately lead to speciation.

In this thesis, I investigated divergence and the outcome of secondary contact between lineages of the tawny dragon, *Ctenophorus decresii*, which differ in morph number and type. *Ctenophorus decresii* is a sexually dimorphic agamid lizard endemic to South Australia, and comprises two genetically distinct and geographically structured lineages: northern and southern. I tested for differences in colour vision between the lineages, which differ in a sexual signal, male throat coloration, particularly in the absence or presence of ultraviolet (UV) reflectance. The northern lineage is colour polymorphic with four discrete throat morphs which lack significant UV reflectance: orange, yellow, orange-yellow (orange centre surrounded by yellow), and grey. Southern lineage males are monomorphic with blue throats and a strong UV reflectance peak. Male throat coloration is an important intraspecific sexual signal, as it is emphasised in territorial and courtship displays. I investigated whether lineages

differ in visual sensitivity to UV wavelengths by measuring retinal opsin protein expression of four cone opsin genes (SWS1, SWS2, RH2, LWS) using droplet digital PCR. I found that lineages did not differ in gene expression of the four opsins and discussed this in the context of conserved visual sensitives in terrestrial systems.

The lineages meet in a contact zone where multi-locus genetic data suggested the presence of hybrids and potential barriers to gene flow. Using extensive field surveys, male phenotype data, genomic single nucleotide polymorphisms (SNPs), and a mitochondrial (mtDNA) marker, I investigated the outcome of secondary contact between the lineages. Furthermore, I captive-bred pure and first generation (F1) hybrid offspring to characterise colour traits independent of exogenous selection. I found that the contact zone is narrow and several generations old with no parental forms or F1 hybrids present. The northern mtDNA haplotype was prevalent in hybrids, and there were high frequencies of backcrossing to the northern lineage but not to the southern lineage, indicating genetic incompatibilities. The northern throat polymorphism was maintained, without any loss of morphs, whereas the southern throat morph was absent. This contrasted with the more intermediate throat phenotype of captive-bred F1 hybrids, particularly in ultraviolet reflectance, suggesting strong selection for the northern throat phenotype within the contact zone.

The viability and fitness of F1 hybrids have consequences for contact zone dynamics, and ultimately whether species boundaries are eroded or maintained. I performed pure and reciprocal cross F1 hybrids in a laboratory setting and measured parental reproductive traits and offspring fitness traits. I found that northern females have a higher reproductive output with more, larger clutches per breeding season and lower embryonic mortality. Although pure and hybrid offspring did not differ in individual fitness traits, hybrids produced from a combination of northern females and southern males exhibited higher fitness in more

categories (i.e. growth rate, bite force, sprint speed). These factors in combination may contribute to the prevalence of northern lineage mtDNA haplotypes in the contact zone.

Finally, I taxonomically separated the northern and southern lineages of *C. decresii* sensu lato on the basis of differentiation in morphology and male coloration, genetic divergence with restricted gene flow, and geographic structuring. This revision results in *C. decresii* sensu stricto (previously southern lineage) and *C. modestus* (previously northern lineage). I evaluated morphological traits of the type specimen of *Amphibolurus modestus* (Ahl 1926), previously a synonym of *C. decresii* sensu lato, and determined that it represented a specimen of the northern lineage. Therefore, I formally re-instated and re-described *Ctenophorus modestus* (Ahl 1926). The addition of this species to the *C. decresii* species group, which now comprises six species, supports the notion that geographic divergence in male coloration is an important component to speciation in this group.

Declaration

This is to certify that:

i. The thesis comprises only my original work towards the degree Doctor of Philosophy except where indicated in the preface;

ii. Due acknowledgement has been made in the text to all other material used;

iii. The thesis is fewer than 100 000 words in length, exclusive of tables, maps, bibliographies and appendices.

Caroline M. Dong

December 2019

Preface

This thesis includes a series of independent publications or manuscripts in preparation. Although the publications are co-authored, I am primary author on all and performed the fieldwork, laboratory work, analyses, and developed the manuscripts. My supervisors, Devi Stuart-Fox, Adnan Moussalli, and Claire McLean contributed to the development of research questions, analyses, and interpretation for all chapters. Adam Elliott assisted with fieldwork and data collection for Chapter 3. Katrina Rankin contributed to animal husbandry and captive breeding for Chapter 4. Greg Johnston and Katrina Rankin contributed to manuscript development for Chapter 5. All co-authors provided feedback on drafts of manuscripts. Published chapters have been included with the permission of all co-authors.

The first chapter was published in the journal *Ecology and Evolution* on 27 September 2019.

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Chapter 1: General introduction

1.1 COLOUR POLYMORPHISM AND SPECIATION

Understanding the mechanisms and processes that generate and maintain biodiversity is central to speciation research. Polymorphic systems provide significant insights into the evolutionary processes generating and maintaining phenotypic diversity within populations, or selective forces promoting divergence between populations. In particular, colour polymorphism, an extreme form of intraspecific phenotypic variation, is the most well-studied form of polymorphism (Huxley 1955; Gray and McKinnon 2007; McLean and Stuart-Fox 2014; Jamie and Meier 2020). The processes generating and maintaining colour polymorphism have been suggested to promote speciation (Gray and McKinnon 2007). Indeed, recent theoretical and empirical research has shown that colour polymorphism is associated with rapid phenotypic evolution (Corl et al. 2010) and accelerated speciation rates (Hugall and Stuart-Fox 2012).

Colour polymorphism is the co-existence of two or more distinct, heritable, morphs within an interbreeding population, with the rarest occurring too frequently to be solely the result of recurrent mutation (Huxley 1955). It is a tractable and appealing area of study because colour traits are easily observed and measured, the genetic and mechanistic basis of pigmentation is well characterised, and colour pattern variation is predicted to be governed by few genes of major effect (i.e. pleiotropy). Colour morphs often differ in other traits in addition to appearance. Coloration is the most visible expression of a suite of co-adapted morphological, behavioural, physiological, and life-history traits, which represent alternative fitness optima (Maynard-Smith 1996; Sinervo and Lively 1996; McKinnon and Pierotti 2010). The existence of morph-specific trait combinations may allow polymorphic species to exist

in spatially and temporally variable environments, have larger ranges, facilitate range expansion and colonisation or founder events (Forsman et al. 2008), all of which may promote speciation (Corl et al. 2010).

The underlying genetic architecture of polymorphism may promote speciation. Co-adapted trait combinations are generated by correlational selection and maintained by frequency-dependent selection (Sinervo & Svensson, 2002). Together, they are predicted to generate tight physical genetic linkage between associated loci or favour pleiotropic loci that control the expression of multiple traits (Sinervo & Svensson, 2002). Consequently, morphs are predicted to be governed by few genes of major effect, and empirical support has been found in a number of systems (Lank et al. 1995; Sinervo et al. 2001; McKinnon and Pierotti 2010; O'Neill and Beard 2010; Wellenreuther et al. 2014; Küpper et al. 2016; Andrade et al. 2019).

Polymorphic species frequently show some degree of geographic variation in the number, type, and/or frequency of morphs present (Van Gossum et al. 2007; Calsbeek et al. 2009; Corl et al. 2010; Runemark et al. 2010; McLean and Stuart-Fox 2014) and geographic colour variation is a well-known indicator of incipient speciation (Price 2008). Non-adaptive (e.g. genetic drift, historical events) or adaptive processes (e.g. spatial variation in selection) may result in population differences in the number, type, or frequency of morphs and/or give rise to monomorphic daughter lineages. The loss of a morph or emergence of a new one may destabilise population dynamics, alter competitive interactions, and change the nature of selection (e.g. frequency-dependent selection) acting on remaining morphs. This may lead to the breakdown of co-adapted trait complexes, evolution of new ones, and increase the probability of speciation. Further, gene flow (e.g. secondary contact) between populations which differ in morphs may also result in the breakdown of trait complexes and generate genetic incompatibilities, ultimately leading to speciation.

1.2 UTILITY OF CONTACT ZONES

The patterns underlying speciation are challenging to untangle because they occur gradually over millions of years. Therefore, research often focuses on contact zones between lineages or groups in an intermediate stage of divergence wherein reproductive isolation is incomplete. Contact zones, also referred to as hybrid zones, are areas where genetically distinct groups meet and hybridise. Contact zones function as natural laboratories, providing an opportunity to directly assess the progress of speciation (i.e. taxonomic status) and form inferences regarding modes of speciation (Hewitt 1988; Barton and Hewitt 1989).

Determining when prezygotic or postzygotic isolating barriers emerge and their relative importance during speciation is fundamental; therefore examining the outcome of hybridisation between lineages in an intermediate stage of divergence is an area of great interest (e.g. Hatfield and Schluter 1999; Jiggins and Mallet 2000; Jiggins et al. 2001; Mendelson 2003; Coyne and Orr 2004; Nosil 2004; Mendelson et al. 2007). Prezygotic mechanisms act to reduce the probability of mating and successful fertilization by a variety of methods, such as: geographical barriers and ecological separation, behavioural, temporal, mechanical incompatibility, and gametic isolation (Dobzhansky 1937). If fertilization occurs, postzygotic mechanisms may still work to prevent the development of viable or fertile offspring via Dobzhansky-Muller incompatibilities between alleles at different loci that are brought together for the first time in hybrids (nuclear-nuclear; Dobzhansky, 1937; Muller, 1942) or cytonuclear incompatibilities, a disruption in intergenomic coadaptation of nuclear and organelle (e.g. mitochondria and chloroplast) genomes that evolved in allopatry (Burton and Barreto 2012; Hill 2015).

The formation of contact zones can be 'primary' when divergence is occurring between adjacent populations of a species, often driven by selection along an environmental gradient (i.e. parapatric speciation) or 'secondary' when geographically isolated groups diverge and subsequently come back into contact (i.e. allopatric speciation). The structure of a contact zone describes the spatial distribution of phenotypes and genotypes, and is influenced by dispersal, hybrid fitness, and the degree of genetic and ecological divergence between parental forms (Endler 1977; Barton and Hewitt 1985; Harrison 1990, 1993). They may be clinal with a smooth transition from one parental form to another across the contact zone, and hybridisation occurring in the centre (Endler 1977). These may be maintained by a balance between dispersal and intrinsic selection against hybrids (i.e. tension zone; Barton and Hewitt 1985, 1989) or by extrinsic selection when hybrid fitness is geographically bounded (Endler 1977). Alternatively, they may be a mosaic of hybrid and parental forms distributed across a heterogeneous habitat (Harrison and Rand 1989; Rand and Harrison 1989; Ross and Harrison 2002; Larson et al. 2013). This may result from variation in environmental resources, or reflect colonization history of parental forms.

More broadly, when previously isolated populations come into secondary contact, differences in sexual phenotypic characteristics may influence the direction and extent of introgression across a contact zone. A divergence in phenotype traits may result in a corresponding divergence in mating preferences (i.e. prezygotic) and restrict gene flow. If prezygotic barriers are weak and hybridisation proceeds, reinforcement may occur via reduced fitness of hybrids if they exhibit a maladaptive intermediate phenotype (Nosil 2003) or possess genetic incompatibilities. Alternatively, an advantageous trait may spread across a contact zone when it confers a fitness benefit to hybrids and/or the other lineage (Stein and Uy 2006; Pardo-Diaz et al. 2012; Baldassarre et al. 2014; While et al. 2015), eventually eroding species barriers.

1.3 THESIS AIMS

In this thesis, I explore divergence and the outcome of secondary contact between colour polymorphic and monomorphic lineages of the tawny dragon lizard (*Ctenophorus decresii*). My general aims are to:

1. Investigate changes in visual sensitivity associated with divergence of a sexual signal and the signalling environment (Chapter 2).
2. Characterise genetic and phenotypic patterns across the naturally occurring contact zone (Chapter 3).
3. Conduct a captive breeding experiment to assess parental reproductive traits and hybrid fitness, to aid in interpreting genetic and phenotypic patterns observed in the contact zone (Chapter 4).
4. Form conclusions regarding the taxonomic statuses of the two lineages of *C. decresii* (Chapter 5).

1.4 STUDY SYSTEM

The tawny dragon, *Ctenophorus decresii*, is a small (mass < 30g, SVL < 90 mm) rock-dwelling agamid lizard endemic to semi-arid regions of South Australia. The species is restricted to rocky terrain, using rock boulders as lookout perches and basking sites, and relying on rock crevices for shelter. A dorsoventrally compressed head and body allow them to retreat safely to crevices when threatened or faced with extreme temperatures, and when inactive at night (Gibbons 1977). They are insectivorous, sparingly consuming vegetation, and predominantly have avian predators (Gibbons 1977; Stuart-Fox et al. 2003). Sexual maturity is reached at >1 year of age (Gibbons 1977) and the species is estimated to have a lifespan of at least five years in the wild (Yewers 2016). Their mating season is in the spring and early summer

(September – December), following emergence from hibernation. They have a predominantly polygynous mating system, where male territories encompass multiple female territories, with low rates of multiple paternity (Hacking et al. 2017).

C. decresii is sexually dimorphic with larger, more colourful males and cryptically coloured females (Figure 1.1). Males exhibit striking variation in throat coloration, which develops before sexual maturity, is heritable, fixed for life, and does not vary with body size (Gibbons and Lillywhite 1981; Stuart-Fox 2002; Rankin et al. 2016). Their brightly coloured throats function as intraspecific sexual signals, emphasised in territorial and courtship displays (Gibbons 1977, 1979; Stuart-Fox and Johnston 2005; Osborne et al. 2012). These movement-based signalling displays consist of dorsolateral compression accompanied by head bobs, limbs waves, tail flicks and coiling, and hindlimb push ups (Ramos and Peters 2016). Females are predominantly grey-brown to orange-brown on the dorsolateral surface with dense patterning and light speckling (Figure 1.1b,d). They have cream coloured throats with varying degrees of grey reticulation, and there may be orange flushes on the belly and gular region during the breeding season.

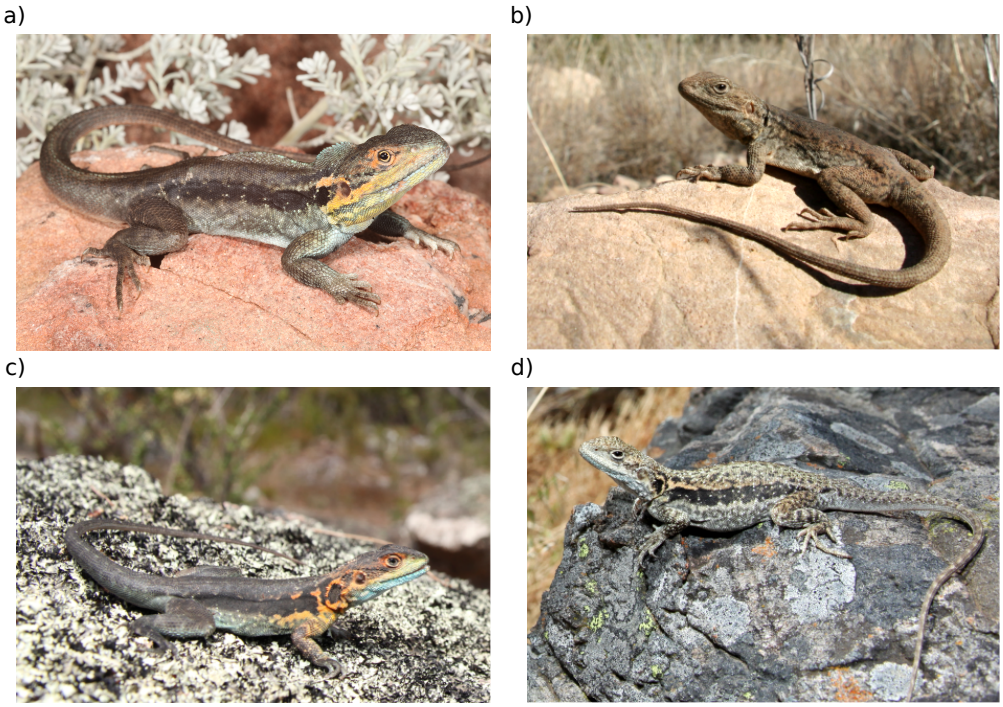


Figure 1.1. Males (a, c) and females (b, d) of the northern (a, b) and southern (c, d) lineages of *C. decresii* (photos Adam Elliott).

Two distinct lineages of *C. decresii* are recognised based on variation in male throat coloration and geographic structure: northern and southern (Houston 1974a; McLean et al. 2014a). The northern lineage is found throughout the Flinders and Olary Ranges, whereas the southern lineage occurs in the Mount Lofty Ranges, Fleurieu Peninsula, and on Kangaroo Island (Figure 1.2). The northern lineage is polymorphic with four discrete throat morphs (orange, yellow, orange centre surrounded by yellow [orange-yellow], and grey) which all lack significant amounts of ultraviolet (UV) reflectance whereas the monomorphic southern lineage has blue throats with a consistent UV reflectance peak (Houston 1974; Teasdale et al. 2013; McLean et al. 2014b; Figures 1.3 and 1.4). Females and juveniles are less distinguishable between lineages.

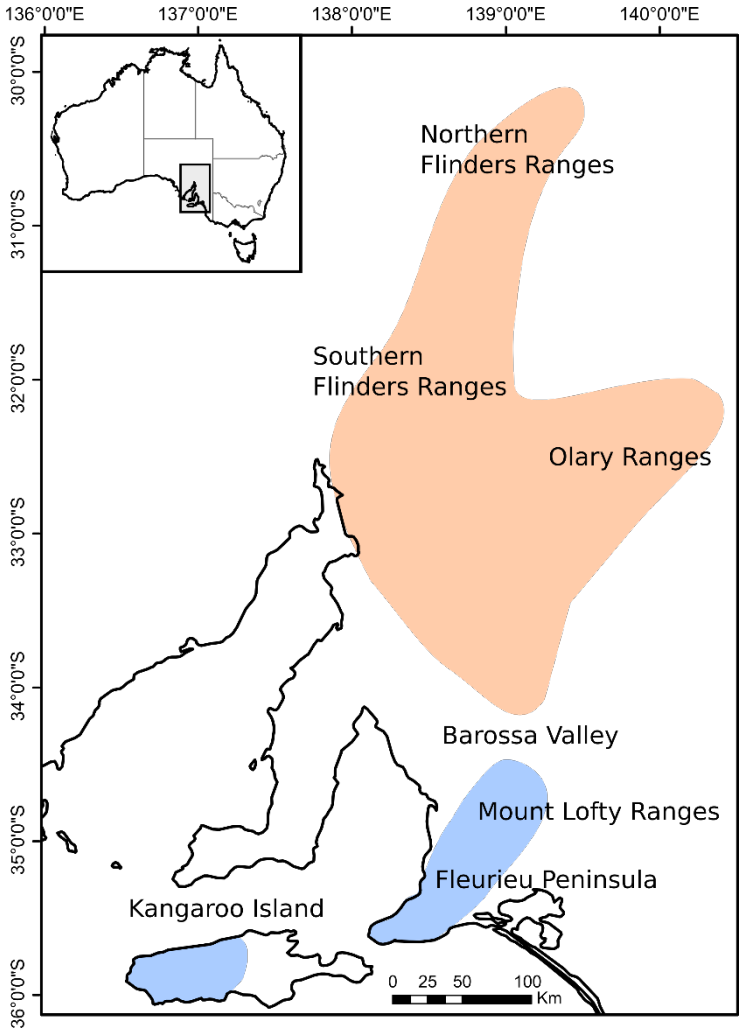


Figure 1.2. Map showing the distributions of the northern and southern lineages of *C. decresii* in shaded orange and blue, respectively, with rocky ranges and geographic areas labelled.

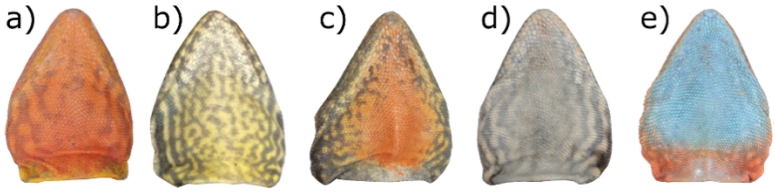


Figure 1.3. Male throat morphs of *C. decresii* found in the northern lineage: a) orange, b) yellow, c) orange-yellow, d) grey; and the southern lineage: e) blue.

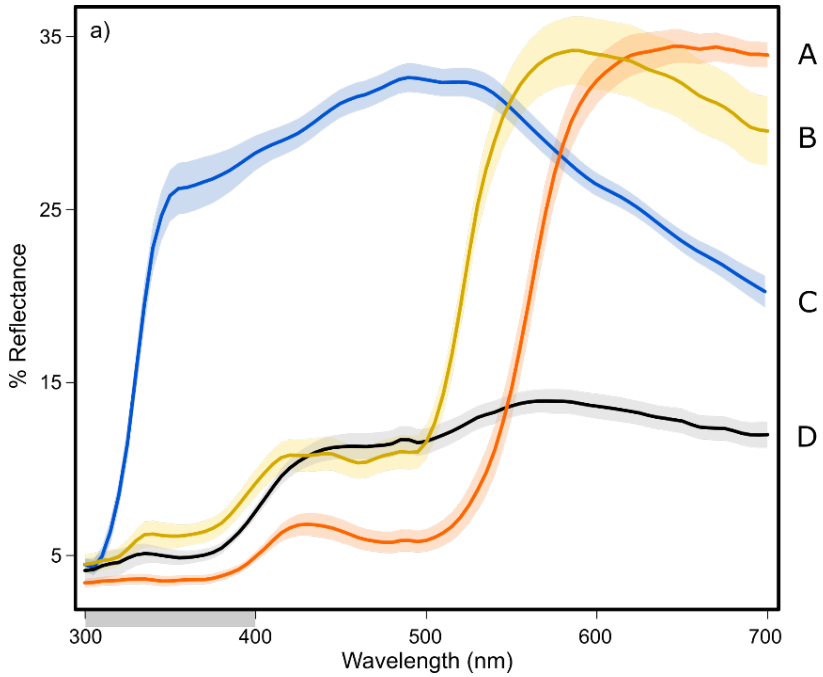


Figure 1.4. Average spectral reflectances of colours found on northern and southern lineage *C. decresii* male throats: a) orange, b) yellow, c) ultraviolet blue, and d) grey. Standard error is shown by shaded region (N = 10 for each colour). Ultraviolet wavelengths are shown with a grey bar on the *x*-axis.

The two lineages meet in a zone of secondary contact near the Barossa Valley, an area of relatively low-lying grassland between the rocky ranges of the north and south (McLean et al. 2014b). Genetic divergence between the lineages (3.7–6.4% net sequence divergence in mtDNA; Mclean et al., 2014) is consistent with contraction to allopatric refugia during the Pleistocene (Byrne 2008). The most likely scenario is that the throat polymorphism evolved once in the ancestor of both *C. decresii* lineages and been subsequently lost in the southern lineage due to changes in selective pressures or by chance, leading to the emergence and fixation of a novel blue morph (McLean et al. 2014b). Secondary contact is likely the result of subsequent post-glacial range expansion. The presence of a naturally occurring contact zone between polymorphic and monomorphic lineages makes this system well-suited for investigating the role of polymorphism in promoting speciation and the evolutionary consequences of secondary contact.

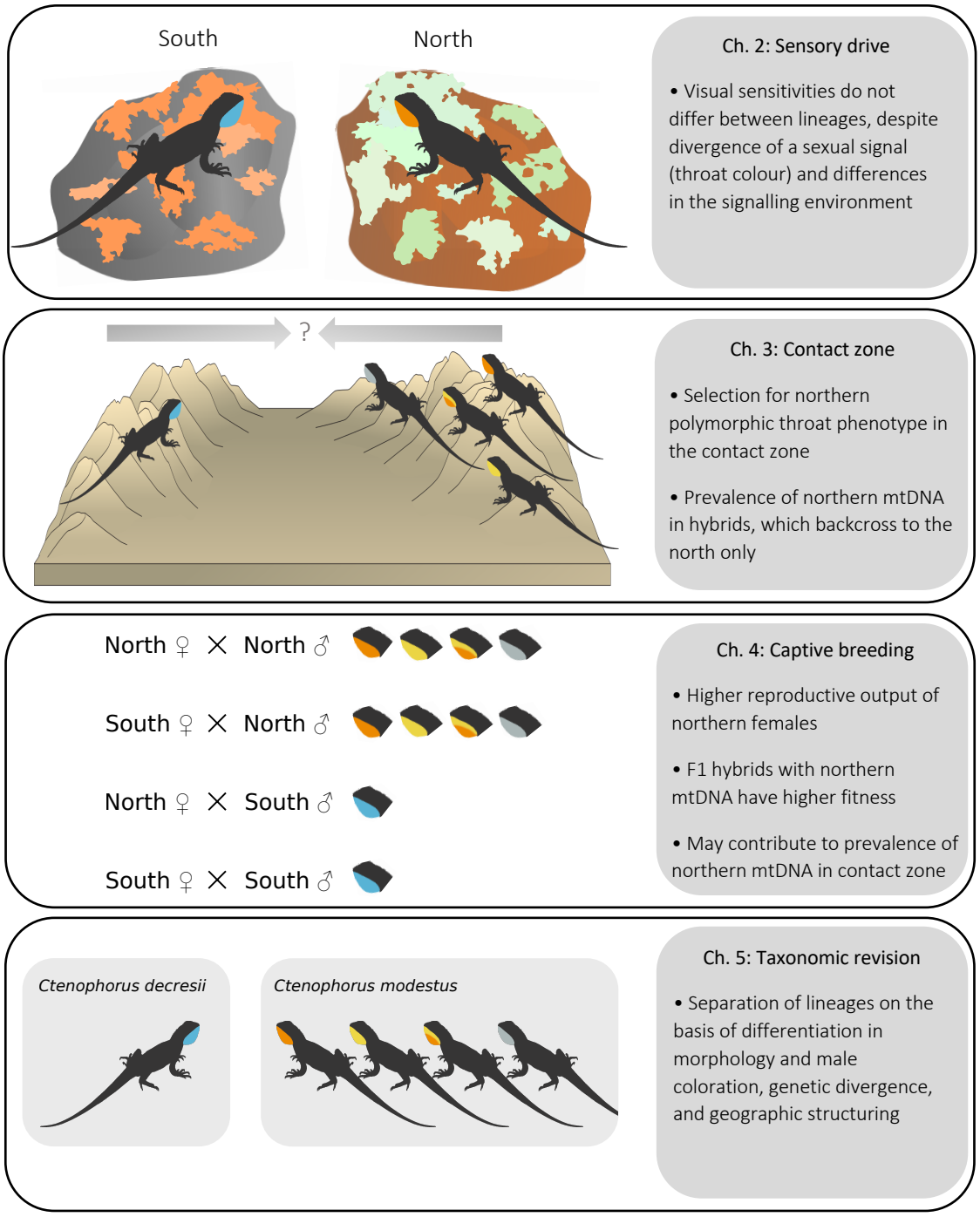


Figure 1.5. Outline of key findings from each data chapter of the thesis.

1.5 THESIS OUTLINE

The overall aim of my thesis is to investigate the evolutionary consequences of secondary contact between colour polymorphic and monomorphic lineages of the tawny dragon lizard, *Ctenophorus decresii*, to increase our understanding of coloration as a driver of speciation (Figure 1.5). My thesis comprises four data chapters that are independent manuscripts, therefore there is some repetition among chapters.

In **Chapter 2**, I assessed the colour vision of the two lineages of *C. decresii* for changes in visual sensitivity associated with divergence of a sexual signal and the signalling environment. The lineages differ in male throat coloration, including ultraviolet (UV) reflectance; additionally, there is evidence that the lineage-specific throat coloration is locally adapted to increase conspicuousness. I investigated gene expression of four retinal cone opsins and found no significant differences in gene expression of individual opsins between the lineages. These results support the hypothesis that diurnal lizards share a highly conserved pattern of tetrachromatic vision, and that sensory drive is rare in terrestrial systems. Instead, males may behaviourally increase the conspicuousness of their throats to conspecifics by selecting backgrounds that contrast with their throat colour.

Chapters 3 and **4** investigate the outcome of hybridisation between lineages, using both field and laboratory studies. I conducted comprehensive surveys of the contact zone from genetically pure southern to northern populations (**Chapter 3**), and used individuals from pure populations at either end for captive breeding (**Chapter 4**). In **Chapter 3**, I characterised genetic and phenotypic patterns across the contact zone to infer traits under selection. Further, I characterised captive-bred F1 hybrid phenotype for comparison with post-F1 hybrid phenotypes found in the field to infer selection on throat phenotype. I found that the contact zone represents a hybrid swarm, with no parentals or F1 hybrids present.

There was evidence of selection for the northern polymorphic throat phenotype within the contact zone. Further, I found evidence for genetic incompatibilities and restricted gene flow. Specifically, the great majority of hybrids had northern mtDNA and genomic introgression was asymmetric, with hybrids backcrossing almost exclusively to the northern lineage.

In **Chapter 4**, I assessed parental reproductive traits and parental effects on offspring fitness traits, interpreting the results in the context of genetic patterns observed in the contact zone. I performed pure and reciprocal F1 hybrid crosses and raised the offspring for up to nine months in order to measure ecologically-relevant fitness traits (e.g. survival, growth rate, bite force, sprint speed). I found that the lineages differ in female reproductive investment and strategy, where northern females had higher fecundity and produced more offspring during a breeding season. Further, I found that parental lineage can influence multiple offspring traits in different ways, providing evidence of the asymmetric fitness of reciprocal cross hybrids when considering the full combination of traits.

Chapter 5 draws on conclusions from previous chapters to provide justification for the taxonomic separation of the northern and southern lineages of *C. decreii*. I performed a morphological analysis of lineages of *C. decresii* and closely related species (*C. fionni* and *C. vadnappa*). I formally described the northern lineage of *C. decresii* sensu lato, and in doing so I resurrected *Ctenophorus modestus* (Ahl, 1926) from synonymy with *C. decresii* sensu stricto, as the original type specimen represents the northern lineage.

Finally in **Chapter 6**, I provide a synthesis of the main findings of my thesis, highlight remaining knowledge gaps, and suggest future directions.

Chapter 2: Conserved visual sensitivities across divergent lizard lineages that differ in an ultraviolet sexual signal



Caroline M. Dong[1, 2], Claire A. McLean[1, 2], Adnan Moussalli[2], & Devi Stuart-Fox[1]

[1]School of BioSciences, The University of Melbourne, Parkville, Victoria, Australia

[2]Sciences Department, Museums Victoria, Carlton Gardens, Victoria, Australia

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Photo: Southern male *Ctenophorus decresii*, Saunders Gorge, South Australia

2.1 ABSTRACT

The sensory drive hypothesis predicts the correlated evolution of signalling traits and sensory perception in differing environments. For visual signals, adaptive divergence in both colour signals and visual sensitivities between populations may contribute to reproductive isolation and promote speciation, but this has rarely been tested or shown in terrestrial species. We tested whether opsin protein expression differs between divergent lineages of the tawny dragon (*Ctenophorus decresii*) that differ in the presence/absence of an ultraviolet sexual signal. We measured the expression of four retinal cone opsin genes (SWS1, SWS2, RH2, LWS) using droplet digital PCR. We show that gene expression between lineages does not differ significantly, including the UV wavelength sensitive SWS1. We discuss these results in the context of mounting evidence that visual sensitivities are highly conserved in terrestrial systems. Multiple competing requirements may constrain divergence of visual sensitivities in response to sexual signals. Instead, signal contrast could be increased via alternative mechanisms, such as background selection. Our results contribute to a growing understanding of the roles of visual ecology, phylogeny, and behaviour on visual system evolution in reptiles.

2.2 INTRODUCTION

When a species exists across habitats of varying physical and structural properties, communication systems may adapt to the local environment to optimise the efficacy of signal transmission and interpretation (Endler 1992, 1993a). This idea is central to the sensory drive hypothesis, which proposes a correlated divergence in signalling traits (e.g. colour), sensory perception (e.g. vision), and mate preferences (Endler and Basolo 1998). The scale and consistency of adaptive divergence in mating signals and perception can influence whether it leads to speciation or the maintenance of colour variation within the species (Chunco et al. 2007; Gray and McKinnon 2007; Seehausen et al. 2008). Despite more than 25 years of research on sensory drive, the majority of work has focused on aquatic systems, where ambient illumination varies substantially and consistently (reviewed in Cummings and Endler 2018). Furthermore, most evidence for tuning of colour vision to different colour signals comes from taxonomic groups that show substantial variation in the number and spectral sensitivities of photoreceptors, such as butterflies and teleost fishes (Bernard and Remington 1991; Carleton et al. 2005; Sison-Mangus 2006; Hoffmann et al. 2007; Briscoe et al. 2010; Miyagi et al. 2012).

In many groups, including birds and reptiles, the number and spectral sensitivities of photoreceptors are highly conserved. However, visual sensitivities can be influenced by the relative proportion of different photoreceptor types (and therefore cone opsin expression) in the retina (reviewed in Carleton 2014). For example, in New World warblers (Parulidae), relative opsin expression is associated with plumage dichromatism and light environment (Bloch 2015). Similarly, a high abundance of ultraviolet (UV) sensitive cones have been associated with the presence of a UV signal in a lizard (Fleishman et al. 2011). Opsin gene expression may evolve more readily in response to varying selection than opsin spectral tuning (i.e. changing the wavelength of peak photoreceptor sensitivity, λmax). However,

evidence for an association between visual signals and colour vision achieved by modifying the relative gene expression of cone opsins is currently limited in terrestrial species (Coyle et al. 2012; Bloch et al. 2015; Yewers et al. 2015; Tseng et al. 2018).

The tawny dragon lizard (*Ctenophorus decresii*; Duméril and Bibron 1837) is a good candidate for examining changes in visual sensitivity associated with divergence of a sexual signal and the signalling environment. The species comprises two genetically and phenotypically distinct lineages which differ markedly in a sexual colour signal, male throat coloration (Figure 2.1). Northern lineage males are polymorphic with four discrete throat morphs: orange, yellow, yellow with an orange centre, and grey (Teasdale et al. 2013), all of which lack significant UV reflectance. By contrast, southern lineage males are monomorphic with UV-blue throats with a consistent UV reflectance peak (McLean et al. 2014b). This throat coloration is prominently displayed during territorial and courtship displays involving head-bobbing and push-ups (Gibbons 1977, 1979; Stuart-Fox and Johnston 2005; Osborne et al. 2012), and is locally adapted to increase conspicuousness against the predominant background colours of native lichen in their respective ranges (McLean et al. 2014a). The northern lineage is primarily found in semi-arid sparsely vegetated habitats, whereas the southern lineage occurs in wetter, temperate, more vegetated habitats (Houston 1974a).

Characteristic of diurnal lizards, *C. decresii* has tetrachromatic vision with UV sensitive (SWS1; 364-383 nm), short-wavelength sensitive (SWS2; 440-467 nm), medium-wavelength sensitive (rod-like cone opsin RH2; 483-501 nm), and long-wavelength sensitive (LWS; 560-625 nm) cone opsins and one rod opsin (RH1) (Yewers et al. 2015). There are no significant differences in the absorption spectra of visual pigments between lineages of *C. decresii*, nor in amino acid sequences of opsin genes (Yewers et al. 2015). However, colour discrimination

may be fine-tuned by differences in the relative proportion of photoreceptor types rather than shifts in their peak wavelength sensitivities. Given the occurrence of locally adapted throat coloration in *C. decresii*, we hypothesized that the lineages may differ in visual sensitivities via the relative expression of cone opsin genes. Specifically, we predicted that the southern lineage would exhibit higher expression of the UV sensitive SWS1 opsin gene due to the UV reflectance peak found on male throats.

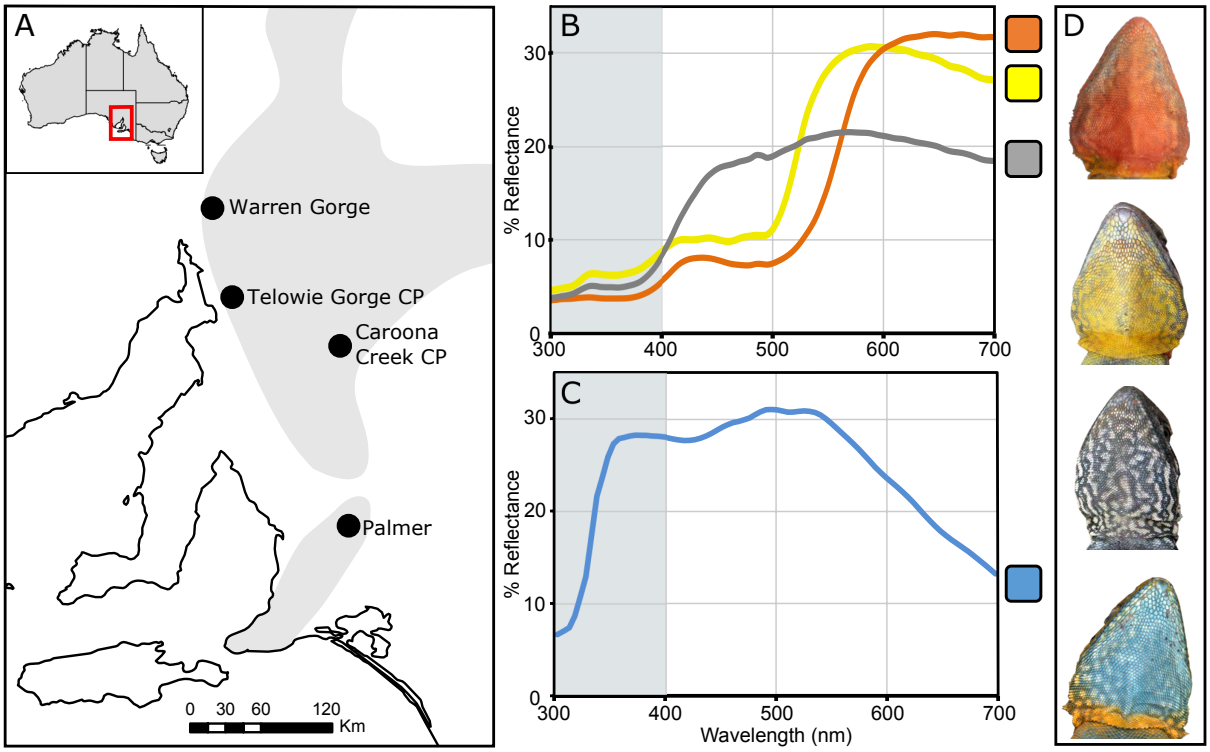


Figure 2.1. Sampling localities and male throat colours of the tawny dragon (*C. decresii*). (a) Map showing localities in the northern and southern lineages. Elevated rocky ranges are shaded in grey. Average reflectance of male throat colours found in the (b) northern lineage: orange, yellow, grey, and in the (c) southern lineage: blue. Ultraviolet wavelengths are highlighted in grey; (d) Representative male throats for each colour.

2.3 METHODS

2.3.1 Animals

We analysed the visual sensitivities of 7 northern lineage (6 males and 1 female) and 9 southern lineage (7 males and 2 females) individuals (Table 2.1). We focused primarily on males because in *C. decresii*, males compete for access to females and opportunities for female male choice appear to be limited, as is generally the case in lizards (Olsson and Madsen 1995; Tokarz 1995; Smith and Zucker 1997; Lebas and Marshall 2001; Lailvaux and Irschick 2006). Male-male interactions are therefore the strongest determinants of mating success in territorial lizards (Gullberg et al. 1997; Stamps and Krishnan 1997; Tokarz 1998; Simon 2011); however, we included a subset of females for comparison. Northern lineage individuals were wild-caught by noosing or by hand from Caroona Creek Conservation Park (longitude: 139.103, latitude: −33.443), Telowie Gorge Conservation Park (longitude: 138.106, latitude: −33.023), or Warren Gorge, South Australia (longitude: 137.995, latitude: -32.183). Southern lineage individuals were captured from Palmer, South Australia (longitude: 139.159, latitude: -34.851) or were hatched from eggs laid by gravid females captured from Palmer and raised in captivity to sexual maturity (> 1 year; Gibbons, 1977). The subjects were housed individually in opaque plastic tubs [55×34×38 cm (L×W×D)] with sand and two terracotta tiles to provide shelter and a basking platform. Lizards were kept in captivity between three months to 1.5 years at The University of Melbourne, Melbourne and Deakin University, Geelong, Australia. They were provided with artificial lighting and seasonal photoperiods to approximate natural conditions, including UV lighting and a heat lamp for thermoregulation. Lizards were fed crickets ad libitum and misted with water three days a week. Although individuals differed slightly in age and period of time in captivity, all were sexually mature at the time of sampling and suitable to investigate consistent differences in visual sensitivities between lineages. This research was conducted with

approval from the Department of Environment, Water and Natural Resources, South Australia (permit nos. E25861-4 and Q26428-3), the Department of Environment, Land, Water and Planning, Victoria (permit nos. 10007000 and 10007751) and with approval by the University of Melbourne Animals Ethics Committee (approval nos. 1312927.1 and 1413220.4), the Wildlife Ethics Committee of South Australia (approval nos. 35/2013 and 25/2015), and the Deakin Animal Ethics Committee (project no. G39-2013).

Table 2.1. Details of all individuals used in the study, including their lineage, sex, capture locality, housing facility, and length of housing (months).

ID	Lineage	Sex	Locality	Housing	Months
NM1	N	M	Telowie Gorge CP	Deakin University	3
NM2	N	M	Telowie Gorge CP	Deakin University	3
NM3	N	M	Caroona Creek CP	Deakin University	3
NM4	N	M	Telowie Gorge CP	Deakin University	3
NM5	N	M	Warren Gorge	The University of Melbourne	3
NM6	N	M	Caroona Creek CP	The University of Melbourne	18
NF1	N	F	Caroona Creek CP	The University of Melbourne	18
SM1	S	M	Palmer	Deakin University	3
SM2	S	M	Palmer	Deakin University	3
SM3	S	M	Palmer	Deakin University	3
SM4	S	M	Captive-bred (Palmer)	The University of Melbourne	6
SM5	S	M	Captive-bred (Palmer)	The University of Melbourne	6
SM6	S	M	Captive-bred (Palmer)	The University of Melbourne	6
SM7	S	M	Captive-bred (Palmer)	The University of Melbourne	6
SF1	S	F	Palmer	The University of Melbourne	8
SF2	S	F	Palmer	The University of Melbourne	4

2.3.2 Quantification of opsin expression

Following humane euthanasia, eyeballs were immediately removed, hemisected, and stored in RNAlater (Ambion Inc., Austin, TX, USA) at -20°C. Whole retinas were dissected for each individual before being disrupted and homogenized using the TissueLyser II with 3mm stainless steel beads (Qiagen, Hilden, Germany). Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified on a 220 TapeStation (RIN scores > 8.0; Agilent, Santa Clara, CA, USA). For each sample, 200 ng of total RNA was reverse-transcribed to cDNA using a qPCRBIO cDNA Synthesis Kit (PCR Biosystems, London, UK) and opsin gene expression was measured using droplet digital PCR (ddPCR), a method of digital PCR that implements a water-in-oil droplet system using the Bio-Rad QX100 system (detailed methods in Hindson et al., 2011). This method provides absolute quantification of copies of the PCR target, without the use of a standard curve, and has been shown to have comparable sensitivity to real-time PCR with the advantages of greater precision and improved reproducibility (Hindson et al. 2013). We used a 25uL reaction mix containing cDNA, primers (900 nM each), probe (250 nM), and ddPCR Supermix for Probes (no dUTP, Bio-Rad, Hercules, CA, USA). Using the QX100 Droplet Generator, this mixture is partitioned into 20,000 droplets and each droplet becomes an independent amplification event. The amplification protocol was as follows: 10 min at 95°C, 40 cycles of 30 sec at 94°C and 1 min at 57°C, followed by 10 min at 98°C, ramp rate set to 2.5°C/s. Following amplification on a standard thermal cycler, droplets are analysed on the QX100 Droplet Reader which counts the number of droplets containing the PCR target (positive) and droplets without (negative) in each sample. These data were analysed with QuantaSoft Analysis Pro v1.0.596 (Bio-Rad, Hercules, CA, USA) which uses a Poisson distribution to determine the absolute template quantity (copies per μL). We then calculated relative opsin gene expression as a percentage of total cone opsin genes expressed for each individual.

We designed primers and probes for the four cone opsin genes (SWS1, SWS2, RH2, LWS; Table 2.2) based on published opsin sequences from retinal transcriptomes of *C. decresii* (Yewers et al. 2015) using Primer3 v4.1.0 (Koressaar and Remm 2007; Untergasser et al. 2012). Additionally, primer binding sites were designed to span an exon-exon junction to avoid amplification of genomic DNA and amplified short fragments (75 - 200 bp). Intron-exon junctions were identified by aligning opsin gene sequences with the draft genome for *C. decresii* (McLean, Stuart-Fox, and Moussalli, unpublished data) using Exonerate v2.2.0 (Slater and Birney 2005). A single mix of primers and probes was used for each opsin gene for the length of the experiment.

Table 2.2. Forward primer, probe and reverse primer sequences for each of the four cone opsins.

Opsin	Forward primer	Reverse primer	Probe
SWS1	ACA GTT CAG GGC TTG CAT TA	TGG AAG AGA CAG AGG AGA CC	/56-FAM/ACC CAT GAC /ZEN/AGA TGA ATC CGA CGT /3IABkFQ/
SWS2	CAA GGC CTC CTC AGT TTA CAA	GAA CTC GAA ACA TCA TCT TCA TCA C	/56-FAM/TGA ACA AGC /ZEN/AGT TCC GCT CCT GTA /3IABkFQ/
LWS	GCT GTC ATT ATC CTC TGC TAC C	CAC TTC CCT TTC AGC CTT CT	/56-FAM/CAG CAA CCG /ZEN/CAC GAA TAG CCA AC/3IABkFQ/
RH2	CTC AAA GAG TTC GTC CCT CTA TAA	GTT CTT GCC ACA GCA GAT TG	/56-FAM/CGT CCT CAT /ZEN/GAA CAA GCA GTT CCG T/3IABkFQ/

2.3.3 Statistical analyses

We used a linear mixed-effects model to examine the effects of lineage on opsin gene expression. Specifically, we had lineage, gene, and their interaction as fixed terms and included months in captivity, lizard ID, and age as random-effect terms (opsin expression ~ lineage + gene + lineage*gene + (1|months) + (1|ID) + (1|age). We used our model to examine the normality of residuals and found significant departure (Shapiro-Wilk normality test, $P < 0.0001$). We log-transformed the data and confirmed normality ($P = 0.30$). The random effect terms each accounted for a negligible amount of variability in the model (< 0.05 total variance). Further, we repeated this analysis on a dataset comprising only males (excluding females; opsin expression ~ lineage + gene + lineage*gene + (1|months) + (1|ID) + (1|age)). Statistical tests were performed in R v3.3 (R Core Development Team 2017) with the packages effects (Fox 2003), lme4 (Bates et al. 2015), and lmerTest (Kuznetsova et al. 2017). We conducted a post hoc power analysis in the program G*Power v3.1.9.4 (Faul et al. 2007, 2009) and determined that the datasets comprising all samples and adult males had powers of 0.94 and 0.86, respectively. A power of 0.80 is generally regarded as an appropriate level of statistical power (Cohen 1988).

2.4 RESULTS

There were no significant differences in gene expression of individual cone opsins between the northern and southern lineages ($P > 0.05$; Table 2.3). For the full dataset, the mean relative gene expression ± SE for the northern lineage was 0.011 ± 0.007, 0.044 ± 0.022, 0.373 ± 0.224, 0.572 ± 0.201 (SWS1, SWS2, RH2, LWS respectively). Likewise, for the southern lineage, mean relative gene expression ± SE was 0.007 ± 0.002, 0.032 ± 0.013, 0.286 ± 0.129, 0.675 ± 0.124 (Figure 2.2a). We found significant differences between opsin genes ($P < 0.0001$; Table 2.3). The relative expression patterns for the four opsin genes were similar between lineages with SWS1 as the lowest expressed gene, followed by SWS2, RH2,

and LWS as the most highly expressed gene (northern = 1 : 4 : 33.9 : 52, southern = 1 : 4.6 : 40.1 : 96.4). Analysis of the subset of males recovered qualitatively similar results with no significant differences in expression of each cone opsin between the northern and southern lineages ($P > 0.05$; Table 2.3). The mean relative expression ± SE for the northern lineage was 0.013 ± 0.006, 0.047 ± 0.021, 0.310 ± 0.164, 0.630 ± 0.142; expression for the southern lineage was 0.007 ± 0.002, 0.036 ± 0.014, 0.286 ± 0.145, 0.671 ± 0.139 (SWS1, SWS2, RH2, LWS respectively; Figure 2.2b). Relative expression patterns were also similar between lineages and to the full dataset (northern = 1 : 3.7 : 24 : 48.7, southern = 1 : 5.2 : 41.6 : 97.4).

Table 2.3. Results of linear mixed-effects models on all individuals (N = 16) and a subset of only males (N = 13), statistically significant values are italicised.

	All Individuals			Males		
	d.f.	F	P	*d.f.*	F	P
Lineage	1	0.59	0.448	1	0.78	0.382
Gene	3	275.72	*< 0.0001*	3	206.94	*< 0.0001*
Lineage x Gene	3	1.0	0.397	3	0.75	0.530

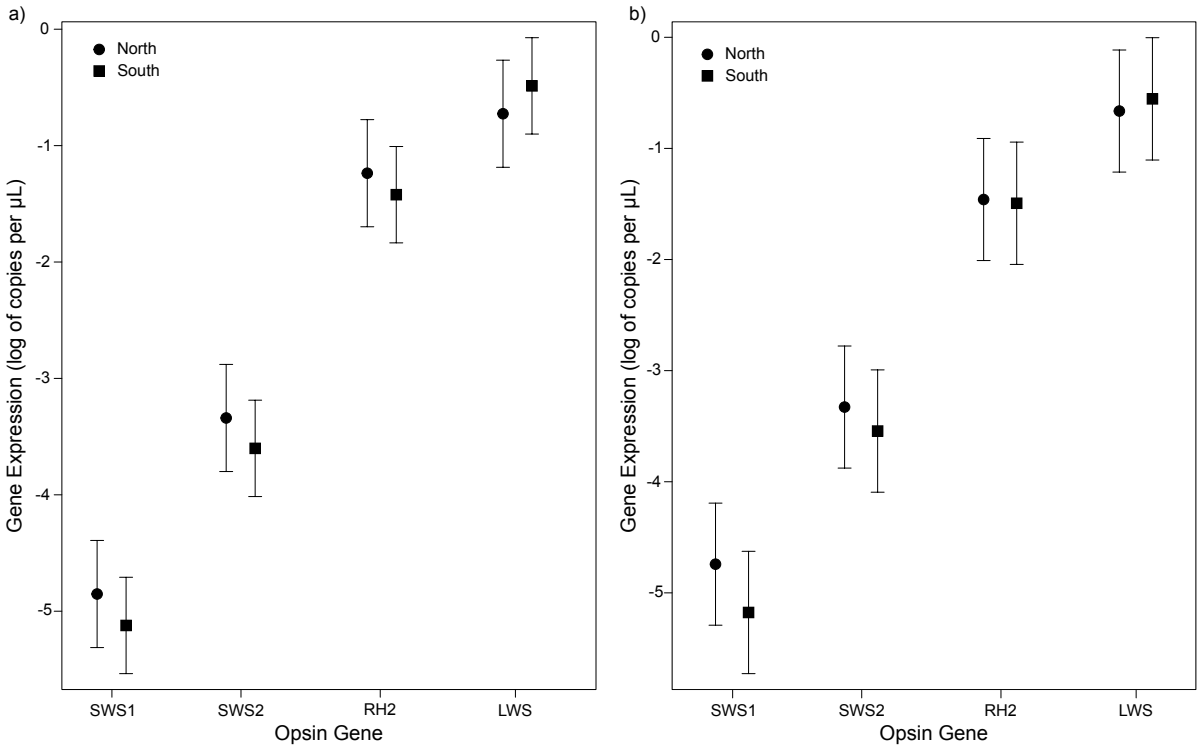


Figure 2.2. Mean relative expressions (copies per µL) of the four cone opsin genes in the northern and southern lineages for a) all individuals and b) a subset of only males. 95% confidence bounds were calculated using parameters estimated from the fitted model.

2.5 DISCUSSION

The coevolution of colour signals and vision is central to the sensory drive hypothesis, a key proposed mechanism of adaptive divergence and speciation. We examined visual sensitivities of *C. decresii* and found no evidence that relative opsin gene expression levels correspond to divergence between lineages which differ in the presence/absence of a UV sexual signal. We found differences in expression between the four opsin genes; this was similar to the relative expression estimated from transcriptome sequencing in *C. decresii* wherein SWS1 and LWS were the lowest and highest expressed genes but small sample sizes limited further statistical inferences (northern = 1 : 2.4 : 2.5 : 31, N = 1; southern = 1.1 : 2.1 : 1 : 19, N = 1; SWS1 : SWS2 : RH2 : LWS; Yewers et al., 2015). A similar hierarchy has been found in the abundance of visual pigments in other squamate taxa (Barbour et al. 2002; Bowmaker et al. 2005; Tseng et al. 2018). Our results support the hypothesis that diurnal lizards share a highly conserved

ancestral pattern of tetrachromatic vision extending into the ultraviolet spectrum as characterised in the families Agamidae (Barbour et al. 2002; Yewers et al. 2015), Chamaeleonidae (Bowmaker et al. 2005), Cordylidae (Fleishman et al. 2011), Dactyloidae (Provencio et al. 1992; Kawamura and Yokoyama 1998; Loew et al. 2002), and Lacertidae (Pérez i de Lanuza and Font 2014). In general, reptiles have retained the set of opsin genes (SWS1, SWS2, RH2, LWS, RH1) inferred to have been present in the ancestral vertebrate (Cronin et al. 2014).

These findings add to the growing body of evidence suggesting that the coevolution of colour signals and visual systems is rare in terrestrial systems (Lind et al. 2017). The strength of selection on visual sensitivities to optimise signal perception is likely to be weaker in terrestrial than aquatic systems because terrestrial light environments are less distinct and more variable over time and space whereas aquatic systems vary more steeply and consistently in background radiance (Levine and MacNichol 1979; Goldsmith 1990; Endler 1993b; Chiao et al. 2000). Although the lineages of *C. decresii* differ in broad aspects of their habitat (aridity and vegetation cover), both prefer brightly lit open perches and irradiance spectra from full sun conditions are similar across northern and southern localities (McLean et al. 2014a). Moreover, the magnitude of changes in irradiance over a diurnal cycle are likely to be much greater than the overall variation in light conditions between lineages (Endler 1993b).

The greater variability of terrestrial environments allows for behavioural adjustments to select backgrounds with spectral properties which optimise signal contrast. In *C. decresii*, adults are almost exclusively found on rocks and the colours of the native rock and lichen differs between lineages. Grey and pink rocks with orange lichen are found in the range of the southern lineage whereas orange rocks with pale green lichen are principally found in

the range of the northern lineage. The throat coloration of each lineage has been shown to be more chromatically conspicuous against the predominant native lichen colour background and less conspicuous against native rock colour backgrounds (McLean et al. 2014a). This suggests that individuals could select a background substrate to maximize contrast or crypsis. There is evidence from various terrestrial taxa of local adaptation and/or that individuals can select or modify their environment to alter their conspicuousness (Endler and Théry 1996; Macedonia 2001; Leal and Fleishman 2002; Heindl and Winkler 2003; Uy and Endler 2004; Endler and Day 2006; Bortolotti et al. 2011; Sicsú et al. 2013; Nafus et al. 2015; Marshall et al. 2016; Klomp et al. 2017; Gunderson et al. 2018). For example, two closely related species of Anolis lizards, *A. cooki* and *A. cristatellus*, differ markedly in dewlap UV-reflectance and have adaptively diverged in microhabitat preference to select light conditions that maximize signal contrast (Leal and Fleishman 2002). However, similar to our findings, there are no significant differences in spectral sensitivity between ecologically diverse species of Anolis with varying dewlap colorations (Fleishman et al. 1997; Loew et al. 2002),

Other ecological factors may generate selection pressures on aspects of the visual system that constrain divergence in response to sexual signals. Visual systems have evolved to accommodate a broad gauntlet of activities critical to survival and fitness. Lizards rely primarily on visual signals at longer distances (López and Martín 2001; López et al. 2002; Whiting et al. 2009) and individuals must detect and interpret many objects in their environment including suitable shelter, potential rivals and mates, predators, and prey. The lineages of *C. decresii* share similar predominantly avian predators and a generalist diet of insects (Gibbons 1977), which often have colour patterns that reflect or absorb selectively in the UV spectrum (Théry and Gomez 2010). Additionally, males of both lineages may need to interpret UV signals in females. In the closely related species *Ctenophorus ornatus*, males prefer females with higher throat UV reflectance (Lebas and Marshall 2000). Female *C.*

decresii have UV-white throats and some exhibit yellow coloration on the throat and sides of the abdomen during the breeding season (Dong and Stuart-Fox, pers. obs.). The function of female coloration in *C. decresii* is yet to be determined, but could indicate receptivity as in other closely related species (Stuart-Fox and Goode 2014). Thus, there may be stronger selection for visual sensitivities that optimise performance across a variety of tasks, than for those tuned to a specific sexually-selected signal.

Visual sensitivities can vary in relation to a range of intrinsic and extrinsic factors, such as carotenoid availability, sex steroids, and light environment. For example, testosterone may regulate opsin expression in the sexually dimorphic green-spotted grass lizard *Takydromus viridipunctatus* (Tseng et al. 2018). However, the great majority of evidence for environmental regulation of opsin expression comes from fish, which experience drastic changes in ambient light environment with habitat changes during ontogeny (Bowmaker and Kunz 1987; Shand et al. 1999, 2002, 2008; Cheng 2004; Cottrill et al. 2009) and even across diurnal cycles (Johnson et al. 2013). It is possible that environmental factors contributed variation to our data; however, there were no differences in opsin gene expression between sexes and these groups do not differ in diet or habitat preferences (Gibbons 1977).

2.6 CONCLUSIONS

In summary, we found no evidence for divergence in visual sensitivities between lineages of *C. decresii* that differ in the presence of a UV sexual signal. Our findings are consistent with weaker divergent selection on visual sensitivities within and between closely related species in terrestrial systems. The lack of divergence in visual sensitivities between lineages of *C. decresii* can likely be attributed to similar selection on colour vision imposed by the abiotic and biotic environment. Instead, male *C. decresii* may increase conspicuousness of their

throat coloration to conspecifics by selecting contrasting backgrounds. By testing for opsin expression divergence in a terrestrial reptile, our study contributes to a growing understanding of broad-scale patterns in the coevolution of signals and sensory systems, and how they differ between taxonomic groups and environments.

2.7 ACKNOWLEDGEMENTS

We are grateful to Madeleine Yewers for access to tissue samples and fieldwork; Katrina Rankin for animal husbandry assistance; Jessica Hacking and Adam Elliott for fieldwork; Franca Casagranda for ddPCR guidance; and the landowners who granted access to their property for data collection. This work was supported by the Australian Research Council (DP1092908 to D.S.-F.).

2.8 APPENDICES

Appendix 2.1. Conserved visual sensitivities across divergent lizard lineages that differ in an ultraviolet sexual signal.

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ORIGINAL RESEARCH

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Conserved visual sensitivities across divergent lizard lineages that differ in an ultraviolet sexual signal

Caroline M. Dong[1,2] | Claire A. McLean[1,2] | Adnan Moussalli[2] | Devi Stuart-Fox[1]

[1]School of BioSciences, The University of Melbourne, Parkville, Victoria, Australia

[2]Sciences Department, Museums Victoria, Carlton, Victoria, Australia

Correspondence
Caroline M. Dong, School of BioSciences, The University of Melbourne, Parkville, Vic. 3010, Australia.
Email: caroline.dong@unimelb.edu.au

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Abstract

The sensory drive hypothesis predicts the correlated evolution of signaling traits and sensory perception in differing environments. For visual signals, adaptive divergence in both color signals and visual sensitivities between populations may contribute to reproductive isolation and promote speciation, but this has rarely been tested or shown in terrestrial species. We tested whether opsin protein expression differs between divergent lineages of the tawny dragon (*Ctenophorus decresii*) that differ in the presence/absence of an ultraviolet sexual signal. We measured the expression of four retinal cone opsin genes (SWS1, SWS2, RH2, and LWS) using droplet digital PCR. We show that gene expression between lineages does not differ significantly, including the UV wavelength sensitive SWS1. We discuss these results in the context of mounting evidence that visual sensitivities are highly conserved in terrestrial systems. Multiple competing requirements may constrain divergence of visual sensitivities in response to sexual signals. Instead, signal contrast could be increased via alternative mechanisms, such as background selection. Our results contribute to a growing understanding of the roles of visual ecology, phylogeny, and behavior on visual system evolution in reptiles.

KEYWORDS

cone opsin, *Ctenophorus decresii*, ddPCR, gene expression, sensory drive, visual ecology

1 | INTRODUCTION

When a species exists across habitats of varying physical and structural properties, communication systems may adapt to the local environment to optimize the efficacy of signal transmission and interpretation (Endler, 1992, 1993a). This idea is central to the sensory drive hypothesis, which proposes a correlated divergence in signaling traits (e.g., color), sensory perception (e.g., vision), and mate preferences (Endler & Basolo, 1998). The scale and consistency of adaptive divergence in mating signals and perception can influence whether it leads to speciation or the maintenance of color variation within the species (Chunco, McKinnon, & Servedio, 2007; Gray & McKinnon, 2007; Seehausen et al., 2008). Despite more than 25 years of research on sensory drive, the majority of work has focused on aquatic systems, where ambient illumination varies substantially and consistently (reviewed in Cummings & Endler, 2018). Furthermore, most evidence for tuning of color vision to different color signals comes from taxonomic groups that show substantial variation in the number and spectral sensitivities of photoreceptors, such as butterflies and teleost fishes (Bernard & Remington, 1991; Briscoe et al., 2010; Carleton, Parry, Bowmaker, Hunt, & Seehausen, 2005; Hoffmann et al., 2007; Miyagi et al., 2012; Sison-Mangus, 2006).

In many groups, including birds and reptiles, the number and spectral sensitivities of photoreceptors are highly conserved.

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However, visual sensitivities can be influenced by the relative proportion of different photoreceptor types (and therefore cone opsin expression) in the retina (reviewed in Carleton, 2014). For example, in New World warblers (Parulidae), relative opsin expression is associated with plumage dichromatism and light environment (Bloch, 2015). Similarly, a high abundance of ultraviolet (UV) sensitive cones have been associated with the presence of a UV signal in a lizard (Fleishman, Loew, & Whiting, 2011). Opsin gene expression may evolve more readily in response to varying selection than opsin spectral tuning (i.e., changing the wavelength of peak photoreceptor sensitivity, λ_{max}). However, evidence for an association between visual signals and color vision achieved by modifying the relative gene expression of cone opsins is currently limited in terrestrial species (Bloch, Morrow, Chang, & Price, 2015; Coyle, Hart, Carleton, & Borgia, 2012; Tseng et al., 2018; Yewers et al., 2015).

The tawny dragon lizard (*Ctenophorus decresii*; Duméril & Bibron, 1837) is a good candidate for examining changes in visual sensitivity associated with divergence of a sexual signal and the signaling environment. The species comprises two genetically and phenotypically distinct lineages which differ markedly in a sexual color signal, male throat coloration (Figure 1). Northern lineage males are polymorphic with four discrete throat morphs: orange, yellow, yellow with an orange center, and gray (Teasdale, Stevens, & Stuart-Fox, 2013), all of which lack significant UV reflectance. By contrast, southern lineage males are monomorphic with UV-blue throats with a consistent UV reflectance peak (McLean, Stuart-Fox, & Moussalli, 2014). This throat coloration is prominently displayed during territorial and courtship displays involving head-bobbing and push-ups (Gibbons, 1979, 1977; Osborne, Umbers, Backwell, & Keogh, 2012; Stuart-Fox & Johnston, 2005) and is locally adapted to increase conspicuousness against the predominant background colors of native lichen in their respective ranges (McLean, Moussalli, & Stuart-Fox, 2014). The northern lineage is primarily found in semi-arid sparsely vegetated habitats, whereas the southern lineage occurs in wetter, temperate, more vegetated habitats (Houston, 1974).

Characteristic of diurnal lizards, *C. decresii* has tetrachromatic vision with UV sensitive (SWS1; 364–383 nm), short-wavelength sensitive (SWS2; 440–467 nm), medium-wavelength sensitive (rod-like cone opsin RH2; 483–501 nm), and long-wavelength sensitive (LWS; 560–625 nm) cone opsins and one rod opsin (RH1; Yewers et al., 2015). There are no significant differences in the absorption spectra of visual pigments between lineages of *C. decresii*, nor in amino acid sequences of opsin genes (Yewers et al., 2015). However, color discrimination may be fine-tuned by differences in the relative proportion of photoreceptor types rather than shifts in their peak wavelength sensitivities. Given the occurrence of locally adapted throat

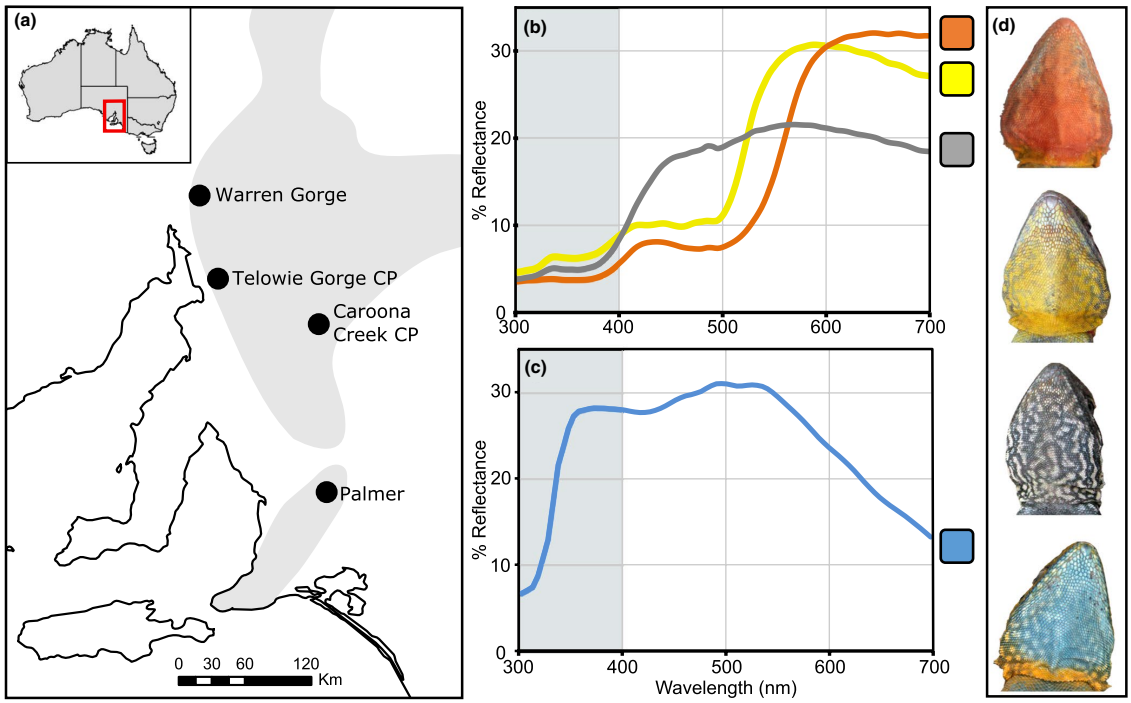


FIGURE 1 Sampling localities and male throat colors of the tawny dragon (*Ctenophorus decresii*). (a) Map showing localities in the northern and southern lineages. Elevated rocky ranges are shaded in gray. Average reflectance of male throat colors found in the (b) northern lineage: orange, yellow, gray, and in the (c) southern lineage: blue. Ultraviolet wavelengths are highlighted in gray; (d) Representative male throats for each color

coloration in *C. decresii*, we hypothesized that the lineages may differ in visual sensitivities via the relative expression of cone opsin genes. Specifically, we predicted that the southern lineage would exhibit higher expression of the UV sensitive SWS1 opsin gene due to the UV reflectance peak found on male throats.

2 | METHODS

2.1 | Animals

We analyzed the visual sensitivities of seven northern lineage (six males and one female) and nine southern lineage (seven males and two females) individuals (Table 1). We focused primarily on males because in *C. decresii*, males compete for access to females and opportunities for female male choice appear to be limited, as is generally the case in lizards (Lailvaux & Irschick, 2006; Lebas & Marshall, 2001; Olsson & Madsen, 1995; Smith & Zucker, 1997; Tokarz, 1995). Male–male interactions are therefore the strongest determinants of mating success in territorial lizards (Gullberg, Olsson, & Tegelstrom, 1997; Simon, 2011; Stamps & Krishnan, 1997; Tokarz, 1998); however, we included a subset of females for comparison. Northern lineage individuals were wild-caught by noosing or by hand from Caroona Creek Conservation Park (longitude: 139.103, latitude: −33.443), Telowie Gorge Conservation Park (longitude: 138.106, latitude: −33.023), or Warren Gorge, South Australia (longitude: 137.995, latitude: −32.183). Southern lineage individuals were captured from Palmer, South Australia (longitude: 139.159, latitude: −34.851) or were hatched from eggs laid by gravid females captured from Palmer and raised in captivity to sexual maturity (>1 year; Gibbons, 1977). The subjects were housed individually in opaque plastic tubs (55 × 34 × 38 cm [L × W × D]) with sand and two terracotta tiles to provide shelter and a basking platform. Lizards were kept in captivity between 3 months to 1.5 years at The University of Melbourne, Melbourne and Deakin University, Geelong, Australia. They were provided with artificial lighting and seasonal photoperiods to approximate natural conditions, including UV lighting and a heat lamp for thermoregulation. Lizards were fed crickets *ad libitum* and misted with water 3 days a week. Although individuals differed slightly in age and period of time in captivity, all were sexually mature at the time of sampling and suitable to investigate consistent differences in visual sensitivities between lineages. This research was conducted with approval from the Department of Environment, Water and Natural Resources, South Australia (permit nos. E25861-4 and Q26428-3), the Department of Environment, Land, Water and Planning, Victoria (permit nos. 10007000 and 10007751) and with approval by the University of Melbourne Animals Ethics Committee (approval nos. 1312927.1 and 1413220.4), the Wildlife Ethics Committee of South Australia (approval nos. 35/2013 and 25/2015), and the Deakin Animal Ethics Committee (project no. G39-2013).

TABLE 1 Details of all individuals used in the study, including their lineage, sex, capture locality, housing facility, and length of housing (months)

ID	Lineage	Sex	Locality	Housing	Months
NM1	N	M	Telowie Gorge CP	Deakin University	3
NM2	N	M	Telowie Gorge CP	Deakin University	3
NM3	N	M	Caroona Creek CP	Deakin University	3
NM4	N	M	Telowie Gorge CP	Deakin University	3
NM5	N	M	Warren Gorge	The University of Melbourne	3
NM6	N	M	Caroona Creek CP	The University of Melbourne	18
NF1	N	F	Caroona Creek CP	The University of Melbourne	18
SM1	S	M	Palmer	Deakin University	3
SM2	S	M	Palmer	Deakin University	3
SM3	S	M	Palmer	Deakin University	3
SM4	S	M	Captive-bred (Palmer)	The University of Melbourne	6
SM5	S	M	Captive-bred (Palmer)	The University of Melbourne	6
SM6	S	M	Captive-bred (Palmer)	The University of Melbourne	6
SM7	S	M	Captive-bred (Palmer)	The University of Melbourne	6
SF1	S	F	Palmer	The University of Melbourne	8
SF2	S	F	Palmer	The University of Melbourne	4

2.2 | Quantification of opsin expression

Following humane euthanasia, eyeballs were immediately removed, hemisected, and stored in RNAlater (Ambion Inc.) at −20°C. Whole retinas were dissected for each individual before being disrupted and homogenized using the TissueLyser II with 3 mm stainless steel beads (Qiagen). Total RNA was extracted using an RNeasy Mini Kit (Qiagen) and quantified on a 220 TapeStation (RIN scores >8.0; Agilent). For each sample, 200 ng of total RNA was reverse-transcribed to cDNA using a qPCRBIO cDNA Synthesis Kit (PCR Biosystems) and opsin gene expression was measured using droplet digital PCR (ddPCR), a method of digital PCR that implements a water-in-oil droplet system using the Bio-Rad QX100 system (detailed methods in Hindson et al., 2011). This method provides absolute quantification of copies of the PCR target, without the use of a standard curve, and has been shown to have comparable sensitivity to real-time PCR with the advantages of greater precision and improved reproducibility (Hindson et al., 2013). We used a 25 ul reaction mix containing cDNA, primers (900 nM each), probe (250 nM), and ddPCR Supermix for Probes (no dUTP, Bio-Rad). Using the QX100 Droplet Generator, this mixture is partitioned into 20,000 droplets and each droplet becomes an independent amplification event. The amplification protocol was as follows: 10 min at 95°C, 40 cycles of 30 s at 94°C and 1 min at 57°C, followed by 10 min at 98°C, ramp rate set to 2.5°C/s. Following amplification on a standard thermal cycler, droplets are analyzed on the QX100 Droplet Reader which counts the number of droplets containing the PCR target (positive) and droplets without (negative) in each sample. These data were analyzed with QuantaSoft Analysis Pro v1.0.596 (Bio-Rad) which uses a Poisson distribution to determine the absolute template quantity (copies per µl). We then calculated relative opsin gene expression as a percentage of total cone opsin genes expressed for each individual.

We designed primers and probes for the four cone opsin genes (SWS1, SWS2, RH2, LWS; Table 2) based on published opsin sequences from retinal transcriptomes of *C. decresii* (Yewers et al., 2015) using Primer3 v4.1.0 (Koressaar & Remm, 2007; Untergasser et al., 2012). Additionally, primer binding sites were designed to span an exon–exon junction to avoid amplification of genomic DNA and amplified short fragments (75–200 bp). Intron–exon junctions were identified by aligning opsin gene sequences with the draft genome for *C. decresii* (McLean, Stuart-Fox, and Moussalli, unpublished data) using Exonerate v2.2.0 (Slater & Birney, 2005). A single mix of primers and probes was used for each opsin gene for the length of the experiment.

2.3 | Statistical analyses

We used a linear mixed-effects model to examine the effects of lineage on opsin gene expression. Specifically, we had lineage, gene, and their interaction as fixed terms and included months in captivity, lizard ID, and age as random-effect terms (opsin expression~lineage + gene + lineage*gene + (1|months) + (1|ID)) + (1|age). We used our model to examine the normality of residuals and found significant departure (Shapiro–Wilk normality test, $p < .0001$). We log-transformed the data and confirmed normality ($p = .30$). The random-effect terms each accounted for a negligible amount of variability in the model (<0.05 total variance). Further, we repeated this analysis on a dataset comprising only males (excluding females; opsin expression~lineage + gene + lineage*gene + (1|months) + (1|ID) + (1|age)). Statistical tests were performed in R v3.3 (R Core Development Team, 2017) with the packages *effects* (Fox, 2003), *lme4* (Bates, Mächler, Bolker, & Walker, 2014), and *lmerTest* (Kuznetsova, 2017). We conducted a post hoc power analysis in the program G*Power v3.1.9.4 (Faul, Erdfelder, Buchner, & Lang, 2009; Faul, Erdfelder, Lang, & Buchner, 2007), and determined that the datasets comprising all samples and adult males had powers of 0.94 and 0.86, respectively. A power of 0.80 is generally regarded as an appropriate level of statistical power (Cohen, 1988).

3 | RESULTS

There were no significant differences in gene expression of individual cone opsins between the northern and southern lineages ($p > .05$; Table 3). For the full dataset, the mean relative gene expression ± *SE* for the northern lineage was 0.011 ± 0.007, 0.044 ± 0.022, 0.373 ± 0.224, 0.572 ± 0.201 (SWS1, SWS2, RH2, LWS, respectively). Likewise for the southern lineage, mean relative gene expression ± *SE* was 0.007 ± 0.002, 0.032 ± 0.013, 0.286 ± 0.129, 0.675 ± 0.124 (Figure 2a). We found significant differences between opsin genes ($p < .0001$; Table 3). The relative expression patterns for the four opsin genes were similar between lineages with SWS1 as the lowest expressed gene, followed by SWS2, RH2, and

TABLE 2 Forward primer, probe, and reverse primer sequences for each of the four cone opsins

Opsin	Forward primer	Reverse primer	Probe
SWS1	ACA GTT CAG GGC TTG CAT TA	TGG AAG AGA CAG AGG AGA CC	/56-FAM/ACC CAT GAC/ZEN/AGA TGA ATC CGA CGT /3IABkFQ/
SWS2	CAA GGC CTC CTC AGT TTA CAA	GAA CTC GAA ACA TCA TCT TCA TCA C	/56-FAM/TGA ACA AGC/ZEN/AGT TCC GCT CCT GTA /3IABkFQ/
LWS	GCT GTC ATT ATC CTC TGC TAC C	CAC TTC CCT TTC AGC CTT CT	/56-FAM/CAG CAA CCG/ZEN/CAC GAA TAG CCA AC/3IABkFQ/
RH2	CTC AAA GAG TTC GTC CCT CTA TAA	GTT CTT GCC ACA GCA GAT TG	/56-FAM/CGT CCT CAT/ZEN/GAA CAA GCA GTT CCG T/3IABkFQ/

	All Individuals			Males		
	df	*F*	*p*	*df*	*F*	*p*
Lineage	1	0.59	.448	1	0.78	.382
Gene	3	275.72	*<.0001*	3	206.94	*<.0001*
Lineage × Gene	3	1.0	.397	3	0.75	.530

TABLE 3 Results of linear mixed-effects models on all individuals (*N* = 16) and a subset of only males (*N* = 13), statistically significant values are italicized

LWS as the most highly expressed gene (northern = 1:4:33.9:52, southern = 1:4.6:40.1:96.4). Analysis of the subset of males recovered qualitatively similar results with no significant differences in expression of each cone opsin between the northern and southern lineages ($p > .05$; Table 3). The mean relative expression ± *SE* for the northern lineage was 0.013 ± 0.006, 0.047 ± 0.021, 0.310 ± 0.164, 0.630 ± 0.142; expression for the southern lineage was 0.007 ± 0.002, 0.036 ± 0.014, 0.286 ± 0.145, 0.671 ± 0.139 (SWS1, SWS2, RH2, LWS, respectively; Figure 2b). Relative expression patterns were also similar between lineages and to the full dataset (northern = 1:3.7:24: 48.7, southern = 1:5.2:41.6:97.4).

4 | DISCUSSION

The coevolution of color signals and vision is central to the sensory drive hypothesis, a key proposed mechanism of adaptive divergence and speciation. We examined visual sensitivities of *C. decresii* and found no evidence that relative opsin gene expression levels correspond to divergence between lineages which differ in the presence/absence of a UV sexual signal. We found differences in expression between the four opsin genes; this was similar to the relative expression estimated from transcriptome sequencing in *C. decresii* wherein SWS1 and LWS were the lowest and highest expressed genes but small sample sizes limited further statistical inferences (northern = 1:2.4:2.5:31, *N* = 1; southern = 1.1:2.1:1: 19, *N* = 1; SWS1:SWS2:RH2:LWS; Yewers et al., 2015). A similar hierarchy has been found in the abundance of visual pigments in other squamate taxa (Barbour et al., 2002; Bowmaker, Loew, & Ott, 2005; Tseng et al., 2018). Our results support the hypothesis that diurnal lizards share a highly conserved ancestral pattern of tetrachromatic vision extending into the ultraviolet spectrum as characterized in the families Agamidae (Barbour et al., 2002; Yewers et al., 2015), Chamaeleonidae (Bowmaker et al., 2005), Cordylidae (Fleishman et al., 2011), Dactyloidae (Kawamura & Yokoyama, 1998; Loew, Fleishman, Foster, & Provencio, 2002; Provencio, Loew, & Foster,

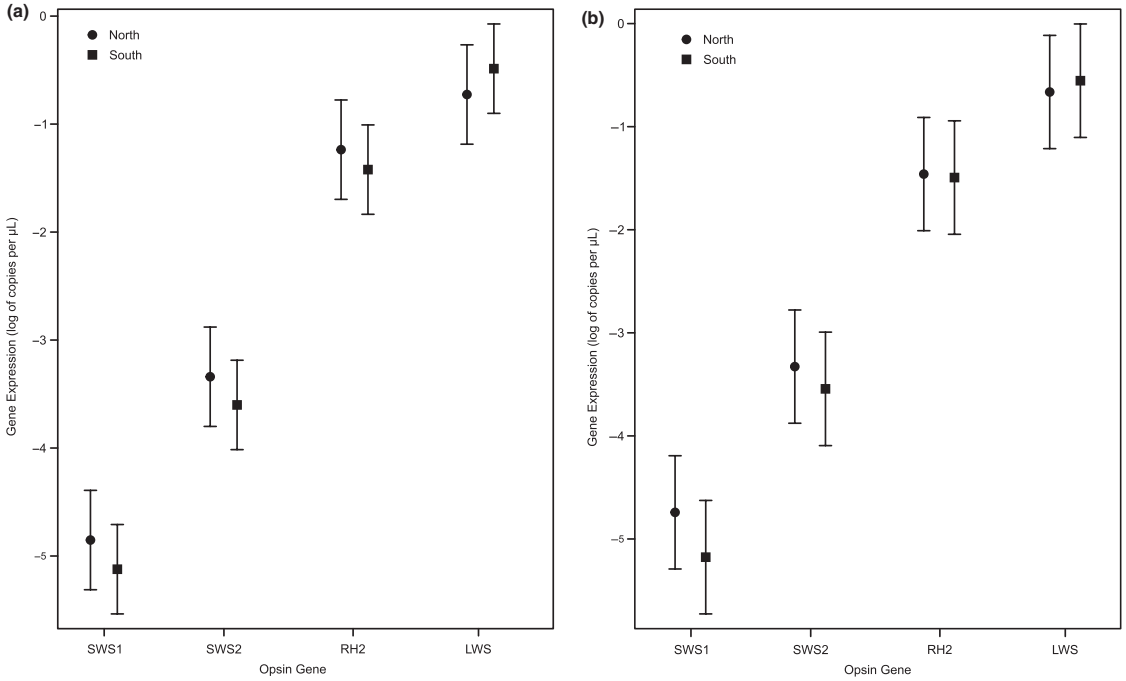


FIGURE 2 Mean relative expressions (copies per µl) of the four cone opsin genes in the northern and southern lineages for (a) all individuals and (b) a subset of only males. 95% confidence bounds were calculated using parameters estimated from the fitted model

1992), and Lacertidae (de Lanuza & Font, 2014). In general, reptiles have retained the set of opsin genes (SWS1, SWS2, RH2, LWS, RH1) inferred to have been present in the ancestral vertebrate (Cronin, Johnsen, Marshall, & Warrant, 2014).

These findings add to the growing body of evidence suggesting that the coevolution of color signals and visual systems is rare in terrestrial systems (Lind, Henze, Kelber, & Osorio, 2017). The strength of selection on visual sensitivities to optimize signal perception is likely to be weaker in terrestrial than aquatic systems because terrestrial light environments are less distinct and more variable over time and space whereas aquatic systems vary more steeply and consistently in background radiance (Chiao, Vorobyev, Cronin, & Osorio, 2000; Endler, 1993b; Goldsmith, 1990; Levine & MacNichol, 1979). Although the lineages of *C. decresii* differ in broad aspects of their habitat (aridity and vegetation cover), both prefer brightly lit open perches and irradiance spectra from full sun conditions are similar across northern and southern localities (McLean, Moussalli, et al., 2014). Moreover, the magnitude of changes in irradiance over a diurnal cycle are likely to be much greater than the overall variation in light conditions between lineages (Endler, 1993b).

The greater variability of terrestrial environments allows for behavioral adjustments to select backgrounds with spectral properties which optimize signal contrast. In *C. decresii*, adults are almost exclusively found on rocks and the colors of the native rock and lichen differs between lineages. Gray and pink rocks with orange lichen are found in the range of the southern lineage whereas orange rocks with pale green lichen are principally found in the range of the northern lineage. The throat coloration of each lineage has been shown to be more chromatically conspicuous against the predominant native lichen color background and less conspicuous against native rock color backgrounds (McLean, Moussalli, et al., 2014). This suggests that individuals could select a background substrate to maximize contrast or crypsis. There is evidence from various terrestrial taxa of local adaptation and/or that individuals can select or modify their environment to alter their conspicuousness (Bortolotti, Stoffel, & Galvan, 2011; Endler & Day, 2006; Endler & Thery, 1996; Gunderson, Fleishman, & Leal, 2018; Heindl & Winkler, 2003; Klomp, Stuart-Fox, Das, & Ord, 2017; Leal & Fleishman, 2002; Macedonia, 2001; Marshall, Philpot, & Stevens, 2016; Nafus et al., 2015; Sicsú, Manica, Maia, & Macedo, 2013; Uy & Endler, 2004). For example, two closely related species of *Anolis* lizards, *A. cooki*, and *A. cristatellus*, differ markedly in dewlap UV reflectance and have adaptively diverged in microhabitat preference to select light conditions that maximize signal contrast (Leal & Fleishman, 2002). However, similar to our findings, there are no significant differences in spectral sensitivity between ecologically diverse species of *Anolis* with varying dewlap colorations (Fleishman et al., 1997; Loew et al., 2002).

Other ecological factors may generate selection pressures on aspects of the visual system that constrain divergence in response to sexual signals. Visual systems have evolved to accommodate a broad gauntlet of activities critical to survival and fitness. Lizards rely primarily on visual signals at longer distances (López & Martín, 2001; López, Martín, & Cuadrado, 2002; Whiting, Webb, & Keogh, 2009), and individuals must detect and interpret many objects in their environment including suitable shelter, potential rivals and mates, predators, and prey. The lineages of *C. decresii* share similar predominantly avian predators and a generalist diet of insects (Gibbons, 1977), which often have color patterns that reflect or absorb selectively in the UV spectrum (Théry & Gomez, 2010). Additionally, males of both lineages may need to interpret UV signals in females. In the closely related species *Ctenophorus ornatus*, males prefer females with higher throat UV reflectance (LeBas & Marshall, 2000). Female *C. decresii* have UV-white throats and some exhibit yellow coloration on the throat and sides of the abdomen during the breeding season (Dong and Stuart-Fox, Personal Observation). The function of female coloration in *C. decresii* is yet to be determined, but could indicate receptivity as in other closely related species (Stuart-Fox & Goode, 2014). Thus, there may be stronger selection for visual sensitivities that optimize performance across a variety of tasks, than for those tuned to a specific sexually selected signal.

Visual sensitivities can vary in relation to a range of intrinsic and extrinsic factors, such as carotenoid availability, sex steroids, and light environment. For example, testosterone may regulate opsin expression in the sexually dimorphic green-spotted grass lizard *Takydromus viridipunctatus* (Tseng et al., 2018). However, the great majority of evidence for environmental regulation of opsin expression comes from fish, which experience drastic changes in ambient light environment with habitat changes during ontogeny (Bowmaker & Kunz, 1987; Cheng, 2004; Cottrill et al., 2009; Shand, Archer, & Collin, 1999; Shand et al., 2008; Shand, Hart, Thomas, & Partridge, 2002) and even across diurnal cycles (Johnson, Stanis, & Fuller, 2013). It is possible that environmental factors contributed variation to our data; however, there were no differences in opsin gene expression between sexes and these groups do not differ in diet or habitat preferences (Gibbons, 1977).

5 | CONCLUSIONS

In summary, we found no evidence for divergence in visual sensitivities between lineages of *C. decresii* that differ in the presence of a UV sexual signal. Our findings are consistent with weaker divergent selection on visual sensitivities within and between closely related species in terrestrial systems. The lack of divergence in visual sensitivities between lineages of *C. decresii* can likely be attributed to similar selection on color vision imposed by the abiotic and biotic environment. Instead, male *C. decresii* may increase conspicuousness of their throat coloration to conspecifics by selecting contrasting backgrounds. By testing for opsin expression divergence in a terrestrial reptile, our study contributes to a growing understanding of broad-scale patterns in the coevolution of signals and sensory systems, and how they differ between taxonomic groups and environments.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

All authors contributed to study design and statistical analysis. CAM contributed to fieldwork. CMD designed primers, conducted ddPCR data collection, and wrote the manuscript. All authors edited and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The dataset and R code are available from Dryad: https://doi.org/10.5061/dryad.0fg8g9h

ORCID

Caroline M. Dong https://orcid.org/0000-0003-3352-4006

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Chapter 3: When polymorphism and monomorphism meet: discordant genomic and phenotypic clines across a lizard contact zone



Caroline M. Dong[1,2], Claire A. McLean[1,2], Adam Elliott[1], Adnan Moussalli[2†], & Devi Stuart-Fox[1†]

[1]School of BioSciences, The University of Melbourne, Parkville, Victoria, Australia

[2]Sciences Department, Museums Victoria, Carlton Gardens, Victoria, Australia

†Joint senior author

Photo: Hybrid male *Ctenophorus decresii*, South Australia (credit Adam Elliott).

3.1 ABSTRACT

Colour polymorphism can promote rapid evolution and speciation, particularly when populations differ in the number or composition of morphs. The tawny dragon, *Ctenophorus decresii*, is a compelling study system in which to examine evolutionary processes and outcomes when polymorphic and monomorphic lineages meet. The species comprises a northern lineage polymorphic for male throat coloration which lacks ultraviolet (UV) reflectance and a monomorphic southern lineage with UV-blue throats. We characterised genomic and phenotypic clines across the contact zone based on single nucleotide polymorphisms, the mitochondrial ND4 gene, and male colour traits, and concurrently assessed the phenotype of captive-bred F1 hybrids. Our results indicate that genomic introgression is asymmetric, with high frequencies of backcrossing to the northern but not southern lineage, accompanied by the prevalence of the northern mtDNA haplotype in hybrids. The clines for throat phenotype are abrupt and displaced to the south, relative to the genetic and dorsolateral phenotype clines. This suggests strong selection for the northern throat phenotype within the contact zone, particularly for the absence of ultraviolet reflectance, given the intermediate throat phenotype in captive-bred F1 hybrids. Our results demonstrate that a polymorphic sexual signal is the target of selection during incipient speciation and provide insight into the microevolutionary processes linking polymorphism and speciation.

3.2 INTRODUCTION

Colour polymorphism, the co-existence of multiple colour morphs within a single interbreeding population (Huxley 1955), is possibly the best studied model of discrete intraspecific phenotypic variation. Recently, there has been great interest in the role of colour polymorphism in promoting rapid phenotypic evolution and speciation, particularly when populations differ in the number, type, and frequency of morphs (West-Eberhard 1986; Gray and McKinnon 2007; Forsman et al. 2008; Corl et al. 2010; Hugall and Stuart-Fox 2012; McLean and Stuart-Fox 2014). However, we lack detailed studies on the microevolutionary processes linking colour polymorphism and speciation. In particular, the genomic and phenotypic consequences of secondary contact between polymorphic and monomorphic lineages remain unclear, especially where the monomorphic lineage represents a novel morph.

Colour morphs often represent alternative fitness optima that differ in a suite of co-adapted morphological, physiological, behavioural, and life-history traits, which allow multiple morphs to co-exist (Maynard-Smith 1996; Sinervo and Lively 1996; reviewed in McKinnon and Pierotti, 2010). These morph-specific trait combinations are generated by correlational selection and maintained by frequency-dependent selection, which together are expected to generate tight physical genetic linkage between associated loci or favour pleiotropic loci that control the expression of multiple traits to ensure optimal combinations (Sinervo and Svensson 2002). Accordingly, morphs are predicted to be governed by few genes of major effect and indeed this has been substantiated in a number of systems (Lank et al. 1995; Sinervo et al. 2001; McKinnon and Pierotti 2010; O'Neill and Beard 2010; Wellenreuther et al. 2014; Küpper et al. 2016; Andrade et al. 2019).

Based on the widespread importance of colour signals in reproductive isolation (Sætre et al. 1997; Boughman 2001; Jiggins et al. 2001; Mavárez et al. 2006; Seehausen et al. 2008) and the underlying genetic architecture of polymorphism (Sinervo and Svensson 2002), there is expected to be a high probability of incompatibility during secondary contact between populations that differ in morph number, type, or frequency. Gene flow may result in the breakdown of adaptive genetic correlations and generate genetic incompatibilities between morphs (Pryke and Griffith 2009). More generally, incompatibilities may arise when coadapted alleles are brought together for the first time in hybrids (Dobzhansky-Muller incompatibilities; Dobzhansky 1937; Muller 1942) or when coadapted nuclear and organelle (e.g. mitochondria and chloroplast) genomes that evolved in allopatry are disrupted (cytonuclear incompatibilities; Burton and Barreto 2012; Hill 2015). Further, divergence in sexual phenotypic characteristics may also be accompanied by behavioural incompatibilities in mating preferences resulting in restricted gene flow and/or selection against hybrids. These phenotypic and genetic incompatibilities together may facilitate the process of speciation between populations which differ in morph frequencies and/or composition.

Here, we investigate the outcome of contemporary contact between colour polymorphic and monomorphic lineages of *Ctenophorus decresii* (tawny dragon; Duméril and Bibron 1837) that differ in both morph number and type. The species comprises two phylogeographically structured lineages which differ in male dorsolateral and throat coloration (Houston 1974; McLean et al. 2014a; Figures 3.1 and 3.2a). The polymorphic northern lineage has four discrete male throat colour morphs that are present in all populations and reflect little to no ultraviolet (UV): orange, yellow, yellow with orange centre, and grey (Teasdale et al. 2013; Figure 3.1a). The black dorsolateral stripe is relatively straight-edged and continuous, sometimes with one distinct blotch near the neck, and with yellow/orange coloration terminating just behind the shoulder (Figure 3.2a; McLean et al. 2013). By contrast, southern

lineage males have blue throat coloration with a strong UV reflectance peak, representing a novel morph (Figure 3.1b; McLean et al. 2014b). Males within an insular population (i.e. Kangaroo Island; "KI") have yellow reticulations in addition to the UV-blue coloration (Figure 3.2a; McLean et al. 2014b). The black dorsolateral stripe is discontinuous with two distinct blotches near the neck and coloration terminating mid-body (McLean et al., 2013). The throat colour polymorphism is likely to have evolved in *C. decresii* and then was subsequently lost in the southern lineage, thereby facilitating the evolution and fixation of the novel blue morph (McLean et al. 2014b). Genetic divergence between the northern and southern lineages (3.7% net sequence divergence in mtDNA) is consistent with range contraction to allopatric refugia during glacial-interglacial Pleistocene cycles (Byrne 2008; McLean et al. 2014b), where differing site-specific selective pressures may have led to the evolution of divergent phenotypes. A small number of genetically admixed individuals have been identified where the lineages meet geographically, suggesting that subsequent post-glacial range expansion has resulted in a secondary contact zone (McLean et al. 2014b).

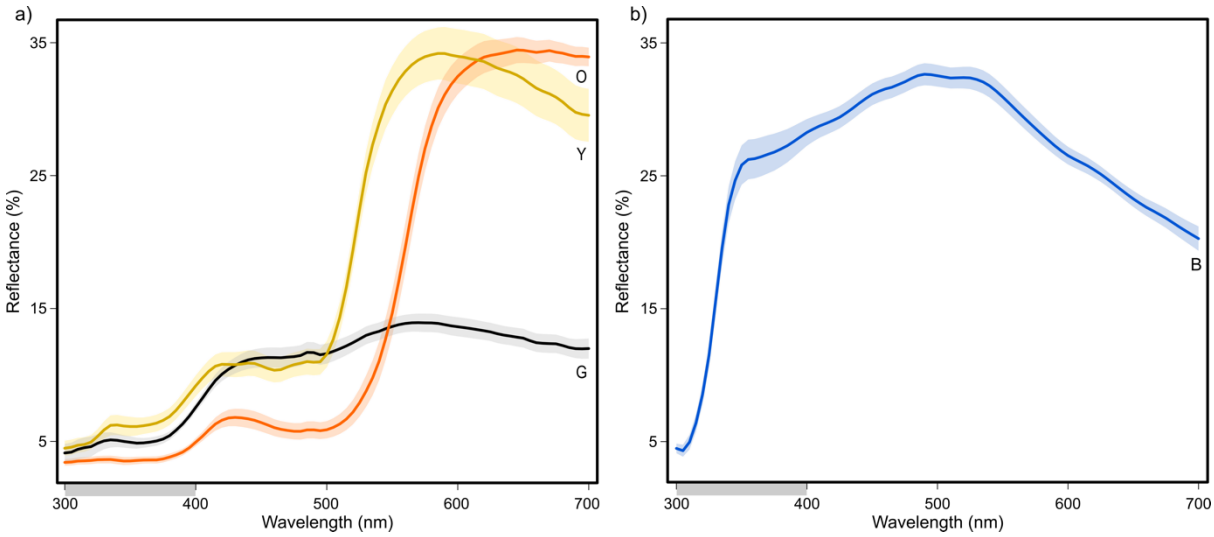


Figure 3.1. Average spectral reflectances of male throat colours found in *C. decresii*. (a) Northern lineage throat colours measured from Southern Flinders Ranges populations: orange (O), yellow (Y), grey (G). (b) Southern lineage ultra-violet blue (B) throat colour measured from Mainland South populations. Standard error shown as shaded region (N = 10 for each colour). Ultraviolet wavelengths are shown with a grey bar on the *x*-axis.

This is a compelling study system to explore the evolutionary consequences of secondary contact between colour polymorphic and monomorphic lineages because their biology is well-characterised. *C. decresii* is sexually dichromatic: females have cream coloured throats with a cryptic dorsal phenotype and males have bright throats with lineage-specific dorsolateral patterning and coloration. Throat and dorsolateral coloration are important intraspecific sexual signals, which are emphasised during complex movement-based territorial and courtship displays involving head-bobbing and push-ups (Gibbons 1977, 1979; Stuart-Fox and Johnston 2005; Osborne et al. 2012; Ramos and Peters 2016). The four morphs of the northern lineage employ alternative behavioural strategies (Yewers et al. 2016) corresponding to differing hormonal profiles (Yewers et al. 2017), and are associated with MHC supertypes (Hacking et al. 2018). Male throat coloration develops before sexual maturity, is fixed for life, and autosomally inherited. The discrete polymorphism is likely controlled by two independently segregating loci, though the extent of orange or yellow within morphs behaves as a quantitative (polygenic) trait (Rankin et al. 2016).

We used a method of double digest restriction-site associated DNA sequencing to identify single nucleotide polymorphisms (SNPs), sequenced the mitochondrial ND4 gene, and characterised male throat and dorsolateral phenotype across a naturally occurring contact zone between the polymorphic northern and monomorphic southern lineages. Using geographic cline analysis, we compared the frequency of these genetic and phenotypic traits along a transect through the contact zone from pure southern to pure northern populations. Furthermore, we conducted captive breeding to investigate F1 hybrid phenotype independent of exogenous selection. Specifically, we examined i) the geographic extent of hybridisation, ii) whether hybridisation is due to recent or earlier admixture, iii) whether hybridisation is bidirectional or asymmetric, and iv) whether there is evidence of selection

on phenotype. We predicted that selection on throat coloration to maintain the northern polymorphism should result in steeper and narrower clines compared to SNP and dorsolateral phenotype clines.

3.3 MATERIALS AND METHODS

3.3.1 Field sampling

Ctenophorus decresii is a small (snout-vent-length [SVL] ≤ 90 mm) diurnal rock-dwelling agamid endemic to South Australia. The northern lineage occurs in the Flinders and Olary Ranges whereas the southern lineage occurs in the Mount Lofty Ranges, Fleurieu Peninsula, and on Kangaroo Island (Figure 3.2b). The contact zone is found in an area of relatively low-lying grassland between the rocky ranges of the parental lineages, coinciding with a change from relatively temperate to more semiarid conditions (Houston 1974a; McLean et al. 2014b). Our sampling design aimed to capture the full geographic range of the species (Figure 3.2b, Appendix 3.1) to reaffirm previously described genetic divergence based on multi-loci sequence data and microsatellites (McLean et al. 2014b). To extend this previous work we intensively sampled 14 sites along a north-south 160 km transect through the contact zone (Figure 3.2c, Appendix 3.2). As the geographic extent of hybridisation was initially unknown, the transect was anchored on either end by two genetically pure southern (sites 1–2) and pure northern (sites 13–14) populations, which were geographically connected along a rock range. Sites were demarcated by geographic distance when possible; however, areas with high densities of sampling were demarcated more finely to allow greater accuracy in analyses. Lizards were captured with a lasso (telescopic pole and fishing line) or by hand and released at the site of capture following data collection. A genetic sample (blood or tail tip stored in 99% ethanol at -20°C) and phenotypic data (described below) were taken

from each individual. We focused on collecting data from adult males (SVL ≥ 65 mm) and collected genetic samples from females opportunistically. Our dataset comprised 234 genetic samples in total from across the full species range (Appendix 3.1). This included 152 genetic samples from the sampling transect, from which we also obtained phenotypic data from up to 233 males along the contact zone transect, depending on the trait (Appendix 3.2).

3.3.2 SNP discovery

Genomic DNA was isolated and purified from blood or tissue using either an E.Z.N.A. Tissue DNA Kit (Omega Bio-tek, Norcross, GA, USA) or a GenCatch Blood and Tissue Genomic Mini-Prep Kit (Epoch Life Sciences, Sugar Land, TX, USA). After quantification using a QIAxpert (Qiagen), we sent between 500 and 1000 ng of DNA at a final concentration of between 50 and 100 ng/uL to Diversity Arrays Technology (Bruce, ACT, Australia) for SNP genotyping using DarTseq, a genome complexity reduction method similar to double digest restriction-site associated DNA (ddRAD) sequencing protocol. Protocols are described in detail for broad applications in Kilian et al., 2012 and specifically for the genus *Ctenophorus* in Melville et al., 2017. Briefly, genomic DNA was digested with two restriction endonucleases (PstI/HpaII), PCR amplified (1 min at 94°C; 30 cycles of 94°C for 20 s, 58°C for 30 s, 72°C for 45 s; final extension at 72°C for 7 min), and sequenced on an Illumina HiSeq2500. 'High-density' DArTseq SNP arrays use 2,500,000 (±7%) reads per sample in marker calling. Two technical replicates of each DNA sample were genotyped to assess reproducibility. DArT analysis pipelines and a calling algorithm (DArTsoft14) were used to process sequences into a matrix of SNP loci. As reference genomes, we used the *C. decresii* draft genome (McLean, Stuart-Fox, and Moussalli, unpublished data) and the fully annotated central bearded dragon genome (*Pogona vitticeps;* Georges et al., 2015). In total, we obtained 172,893 SNPs from 234 individuals with 41.54% missing data. Using the R package *dart*R (v1.1.6, Gruber et al. 2018), we filtered this dataset conservatively with thresholds of

100% reproducibility, ≤1% missing data per locus, ≤10% missing data per individual (four individuals removed), and also removed loci that deviated from Hardy-Weinberg Equilibrium ($P < 0.05$). This resulted in a dataset of 1,333 SNPs from 230 individuals with 0.26% missing data.

3.3.3 Mitochondrial DNA

We sequenced an 850 base-pair region of the mitochondrial genome which included the NADH dehydrogenase subunit four (ND4), and tRNAhis, tRNAser and tRNAleu regions using the primers ND4F (Forstner et al. 1995) and tRNA-Leu (Scott and Keogh 2000) for a subset of 74 individuals from sites across the contact zone transect (Appendix 3.1). PCR was performed in 25μL reactions containing 12.5μL GoTaq Hot Start Master Mix (Promega, Madison, Wisconsin, USA), 1μL forward and reverse primer (10μM), and 1μL DNA. Cycling conditions included an initial denaturation (95°C for 4 min) followed by 35 cycles total of denaturation (95°C for 45 s), annealing (10 and 25 cycles of 45 s at 56°C and 54°C, respectively), and extension (72°C for 1.5 min) with a final extension period of 72°C for 7 min (McLean et al. 2014b). PCR products were submitted to the Australian Genome Research Facility (Melbourne, Victoria, AUS) for purification and forward and reverse sequencing on an Applied Biosystems 3730xl DNA Analyzer. Sequences were edited and aligned with additional ND4 sequences of northern and southern lineage *C. decresii* (McLean, Stuart-Fox, et al., 2014) in Geneious (v6.1.7; Kearse et al. 2012). Haplotype networks were constructed using the statistical parsimony algorithms of TCS (v1.21; Clement et al. 2000).

3.3.4 Quantifying population structure and admixture

We examined genome-wide patterns of divergence using the Bayesian model-based clustering approach implemented in STRUCTURE (v2.3.4; Pritchard et al. 2000). After an

initial burn-in of 500,000 Markov chain Monte Carlo (MCMC) generations, we ran 500,000 generations with 10 independent replicates for K values ranging from 1 to 5 under the "admixture" model with correlated allele frequencies and prior population information, all other parameters were kept at default, and convergence of runs was visually assessed. We used STRUCTURE HARVESTER (Earl and VonHoldt 2012) to identify the optimum value of K for our dataset and summarised results across the selected value of K using CLUMPP (v1.1.2; Jakobsson and Rosenberg 2007). We used the Bayesian estimation of the *q*-value (proportion of an individual's genome inherited from each parental population) with 95% confidence intervals (CI) as a hybrid index. Individuals were identified as pure parentals or putative hybrids based on their hybrid index and 95% CI: an individual was classified as parentals if *q* was less than 0.1 (i.e. North) or greater than 0.9 (i.e. South), or of mixed ancestry if *q* was between 0.1 and 0.9.

We used previously identified population groups within each parental lineage to further examine population structure (North: Northern Flinders Ranges [NFR], Southern Flinders Ranges [SFR], Olary Ranges [OR]; South: Mainland South [MS], Kangaroo Island [KI]; McLean et al. 2014b) and Contact Zone (CZ). We calculated expected and observed heterozygosity, the number of alleles and allelic richness, estimated population pairwise F_{ST}, and the inbreeding coefficient (F) for each population using the R packages *dartR* (v1.1.6; Gruber et al. 2018) and *adegenet* (v2.1.1; Jombart and Ahmed 2011). We then performed a principal coordinate analysis (PCoA) using *dartR* to visualize pairwise genetic distances between individuals.

3.3.5 Hybrid classification

We subsampled our SNP dataset to obtain a dataset of 200 loci with the highest average polymorphic information content values using the R package *dartR* (v1.1.6; Gruber et al.

2018). The dataset was analysed three times in parallel, each with a total of 100,000 MCMC generations after an initial burn-in of 50,000 generations, using the program NewHybrids (v1.1 Anderson & Thompson, 2002) and the R package *parallelnewhybrid* (Wringe et al. 2017). We used the program to determine the probability that individuals within the contact zone belonged to one of the six genotypic classes resulting from the first two generations of hybridisation: parental forms (P1 and P2), F1 (P1 × P2), F2 (F1 × F1), and backcrossed (F1 × P1/P2). Although it is not always possible to unambiguously assign individuals into discrete classes due to the continuum that may be found in wild populations, classification may be suggestive of situations where contact is very recent or where F1 hybrids are infertile based on the absence of F2 hybrids and backcrosses, or where contact is several generations old based on the absence of pure parentals and F1 hybrids.

3.3.6 Captive breeding and testosterone-induced expression of colour traits

To evaluate F1 hybrid phenotype independent of extrinsic selection pressures, we conducted captive breeding using 36 pure southern lineage individuals (female = 17, male = 19) collected from cline site 1 (southernmost site, longitude: 139.103°, latitude: -33.443°) and 41 pure northern lineage individuals (female = 20, male = 21) from cline site 14 (northernmost site, longitude: 139.103°, latitude: -33.443°; Appendix 3.2, Figure 3.2c) to produce reciprocal cross hybrids, in addition to pure lineage offspring for comparison. Male and female pairs were housed together until the female was visibly gravid, eggs were collected following oviposition and incubated until hatching. Offspring were housed individually and reared under laboratory conditions (total = 138; southern = 11, northern = 54, hybrids = 73). We genotyped adults and offspring at five microsatellite loci (Am41, Cp10, Ctde45, Ctde05, Ctde12; Schwartz et al. 2007; McLean et al. 2014b) and confirmed paternity using a maximum likelihood approach in CERVUS (v3.0.7; Marshall et al. 1998; Kalinowski et al. 2007). For

all offspring, we induced throat colour expression by artificially elevating testosterone levels at nine months of age in both sexes (female = 64, male = 74). Females can be reliably induced to express discrete throat morphs found in males using testosterone because throat coloration is autosomally inherited; testosterone may influence the intensity of coloration but morphs remain objectively classifiable (Rankin and Stuart-Fox 2015). Despite testosterone elevation, dorsolateral patterning and coloration remained cryptic therefore dorsolateral phenotype was analysed only for male offspring. See Appendix 3.7 for full details of breeding, rearing, genotyping, and throat colour expression procedures.

3.3.7 Analysis of phenotypic data

To characterise the phenotype of wild-caught males and captive-bred F1 hybrids, we took standardised ventral (i.e. throat) and dorsal photographs and spectral reflectance measurements of throat colours. Photographs were taken using a Canon EOS 600D digital camera, saved in raw format, and included an Xrite colour standard and ruler for scale (Stevens et al. 2007). Images were converted to 8-bit tiff files using Digital Photo Professional (v4.5.0.0; Canon Inc.), linearised with respect to radiance, and equalised against a grey standard (Stevens et al. 2007; as per Smith et al. 2016). We then performed a segmentation analysis (detailed in Teasdale et al. 2013) to extract the proportion of each colour present on the throat (i.e. orange, yellow, blue), classify throats into existing morphs (orange, yellow, orange-yellow, grey, or blue), and to quantify the proportion of orange and yellow colouration present on the head and neck from dorsal photographs. Image analysis was conducted using custom code written in MATLAB (The MathWorks, Inc., Natick, MA, USA; see Teasdale et al. 2013). We also manually quantified the number of distinct breaks in the dorsolateral stripe near the neck by visually inspecting all photographs of the dorsal region.

We took spectral reflectance measurements (300–700 nm) to quantify variation in UV reflectance, using an Ocean Optics (Dunedin, FL, USA) Jaz portable spectrometer. The probe was held at a constant angle (45°) and distance from the surface of the skin. Measurements were expressed relative to a Spectralon 99% diffuse white reflectance standard (Labsphere, Inc., North Sutton, NH, USA). At least two measurements were taken of each colour present on the throat, then averaged. For each throat colour patch, we estimated the receptor quantum catch (QC), the stimulation of retinal photoreceptors, for the violet to UV wavelength sensitive (UVS) photoreceptor because we were specifically interested in the diagnostic UV-blue component of throat coloration. The R package *pavo* (v1.3.1; Maia et al. 2018) was used to process reflectance measurements.

We performed principal component analyses (PCA) to explore variation in throat coloration variables (proportion of orange, proportion of yellow, proportion of blue) and dorsolateral phenotype variables (proportion orange, proportion of yellow, breaks in the dorsolateral stripe) in the R package *FactoMine*R (v1.4.1; Lê et al. 2008). In each, the lineages were differentiated along the first dimension (PC1) and this was used to collapse variables into a single measure of overall phenotype and retained for use in subsequent cline analyses. The quantum catch of the UV sensitive photoreceptor (QC_{UVS}) was used singly in cline analyses to assess its frequency across the contact zone independent of throat colour morph. Due to missing data, 204 individuals were used in throat phenotype analyses whereas 233 individuals were used in dorsolateral phenotype analyses. Linear discriminant analyses were performed on pure individuals in the R package *MASS* (v7.3.50; Ripley 2002) using all throat phenotype variables (proportion of orange, yellow, and blue, and QC_{UVS}) and dorsolateral phenotype variables as independent variables.

3.3.8 Geographic cline analysis

We used the R package *HZAR* (v0.2-5, Derryberry et al. 2014) to fit a series of equilibrium geographic cline models (Szymura and Barton 1986, 1991; Gay et al. 2008) to both the molecular and phenotypic datasets, and in all cases using 1,000,000 MCMC generations following an initial burn-in of 100,000 generations. Specifically, we estimated cline models for the hybrid index (H_{Index}; Bayesian *q*-value based on 1,333 loci), mtDNA haplotype frequencies, quantum catch of the UV wavelength sensitive receptor (QC_{UVS}), PC1 for throat coloration (T_{PC1}), and PC1 for dorsolateral phenotype (D_{PC1}).

HZAR uses logistic regression equations to fit sigmoidal clines in a maximum-likelihood framework and describes cline centre (*c*) as the position where estimated trait frequency is 0.5 and width (*w*) as 1/slope at the cline centre. For molecular clines, we compared 15 models that varied in the fitting of trait interval (*p*; minimum and maximum frequencies at tails; fixed at 0 and 1, observed values, estimated values) and exponential decay tails (none, right only, left only, mirrored, both estimated separately). These 15 models were compared to an additional null model. For phenotypic trait data we compared models that varied in the fitting of exponential decay tails (none, right only, left only, mirrored, both estimated separately) and estimated mean and variance on the ends and in the centre. All models estimated cline centre and width as well as tail parameters if applicable (δ, distance from the centre to the tail; τ, tail slope). QC_{UVS} and T_{PC1} were normalised to values between 0 and 1 for plotting purposes.

To address model selection uncertainty, we conducted model averaging in an Akaike's information criterion (AIC) framework. We calculated the Akaike weight (AIC*w*), a value between 0 and 1 which can be interpreted as the probability of being the best approximating model (Burnham and Anderson 2002). Models were then ranked by AIC*w* downwards until

the cumulative AIC*w* reached 0.95 to attain a 95% confidence set of best-ranked models (Lukacs et al. 2010; Symonds and Moussalli 2011). We recalculated the AIC*w* of models in the 95% confidence set and used those to calculate the model-averaged estimate of parameters (e.g. centre and width). We produced model-averaged clines by first multiplying the estimate for a given distance interval by the AIC*w*. This was done for each model within the 95% confident set of models. The AIC model averaged estimate was then obtained by summing up weighted estimates for each distance interval. Similarly, we multiplied the minimum and maximum estimate bounds by the AIC*w*, followed by summation to represent the credible region. Finally, the coincidence of cline centres was assessed by comparing model-averaged maximum-likelihood derived CIs (two log-likelihood unit support limits) of cline centres, with non-overlapping CIs considered statistically supported differences. Akaike weights were calculated using the R package MuMIn (v1.43.6; Bartoń 2018).

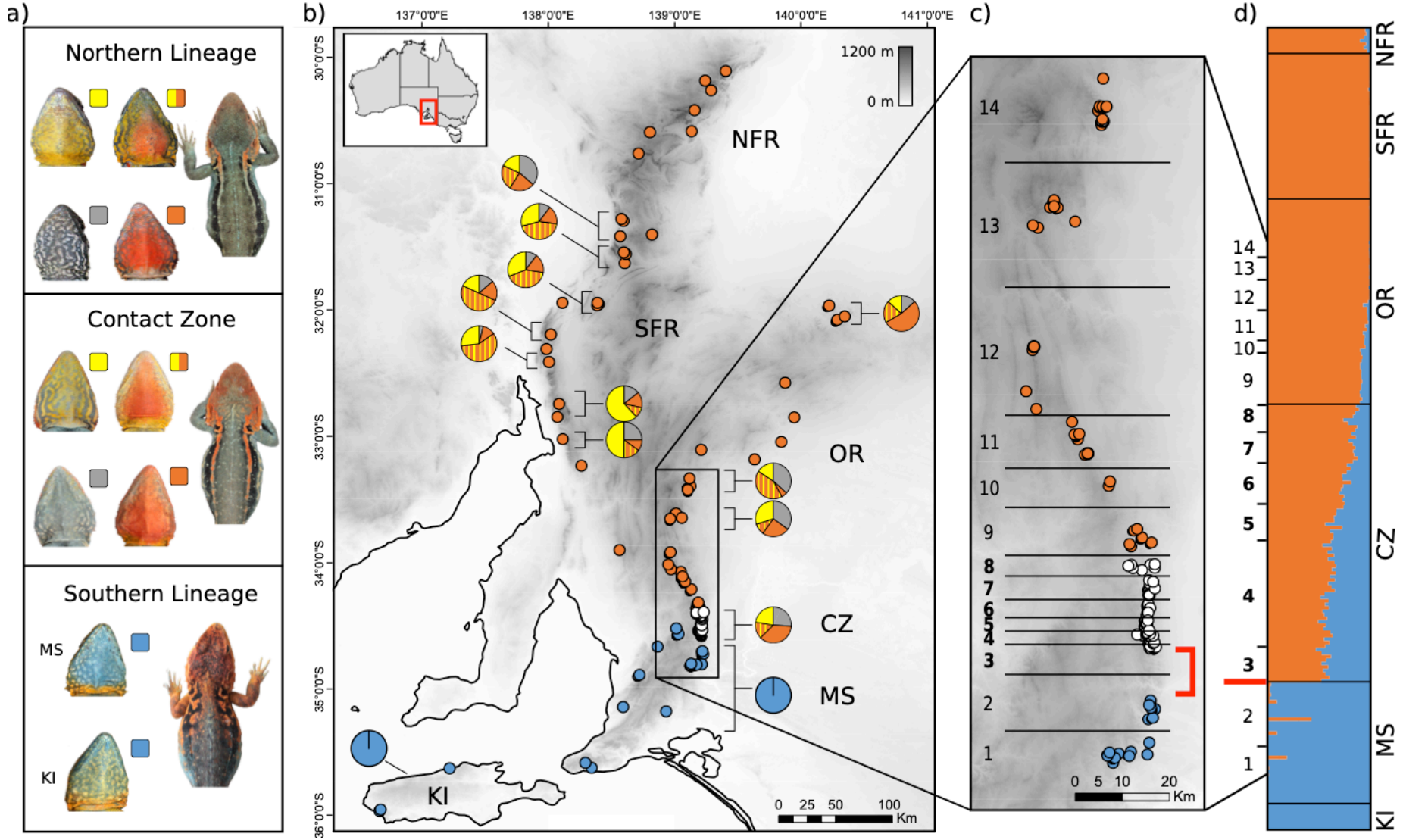

a)
Northern Lineage
Contact Zone
Southern Lineage
MS
KI
b)
137°0'0"E
138°0'0"E
139°0'0"E
140°0'0"E
141°0'0"E
30°0'0"S
31°0'0"S
32°0'0"S
33°0'0"S
34°0'0"S
35°0'0"S
36°0'0"S
1200 m
0 m
NFR
SFR
OR
CZ
MS
KI
0 25 50 100 Km
c)
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0 5 10 20 Km
d)
NFR
SFR
OR
CZ
MS
KI

Figure 3.2. Geographic variation in throat colour morphs and genetic structure between lineages of *C. decresii*. (a) Representative throats (North: yellow, orange-yellow, grey and orange; South: blue) and dorsolateral phenotypes found in each lineage and the contact zone. (b) Map showing sampling localities of individuals used in genetic analyses (Northern Flinders Ranges [NFR], Southern Flinders Ranges [SFR], Olary Ranges [OR], Mainland South [MS], and Kangaroo Island [KI], and genetically admixed individuals (white circles; $0.1 < q < 0.9$). Pie charts indicate relative frequencies of male throat colour morphs. Proportions for the Southern Flinders Ranges and northernmost population in the Olary Ranges are adapted from McLean et al. 2015. Throat colour morph data are not available for the Northern Flinders Ranges. Elevation in meters is represented by grey shading on the scale bar. (c) An enlarged map section showing sites across the contact zone transect used for geographic cline analyses; (d) Results of a Bayesian analysis of ancestry in the program STRUCTURE (K = 2). Each horizontal bar represents an individual and individuals are ordered by latitude and population. The proportion of blue and orange represents the proportion of southern and northern ancestry in each individual. A density trough between sites 2 and 3, where surveys did not recover any individuals, is indicated in red in panels B and C.

3.4 RESULTS

3.4.1 Population structure and admixture

We found distinct population structure across the species range with the most prominent divide between the northern and southern lineages and evidence for the presence of hybrids in the contact zone. Based on the STRUCTURE analysis, only two clusters were identified, corresponding to the major split between the northern and southern lineages (Figure 3.2d, Appendix 3.3). The *q*-values ranged from 0.999 (pure southern) to 0.001 (pure northern). Across the sampling transect, individuals from the southernmost sites 1–2 had a high probability of belonging to the southern genetic cluster ($q \geq 0.90$; mean ± SD = 0.99 ± 0.02) with the exception of two admixed individuals ($q = 0.81$ and 0.57). All individuals from the northernmost sites 9–14 had a high probability of belonging to the northern genetic cluster ($q \leq 0.10$; mean ± SD = 0.04 ± 0.03). Hybrids were identified within sites 3–8 where all individuals were of mixed ancestry and exhibited a highly skewed continuum of *q*-values ranging from 0.53 to 0.11. These sites will hereinafter be referred to as 'the contact zone', encompassing a distance of approximately 22.5 km. We also identified a density trough of approximately 12 km between sites 2 and 3 (Figure 3.2c–d) across which the genotype changes abruptly and multi-year surveys recovered no individuals. With the exception of two individuals at site 3, all admixed individuals in sites 3–13 had northern lineage mtDNA haplotypes. The southern lineage mtDNA haplotype was fixed in sites 1–2, including the only two admixed individuals identified in the geographic range of the southern lineage (Appendix 3.8).

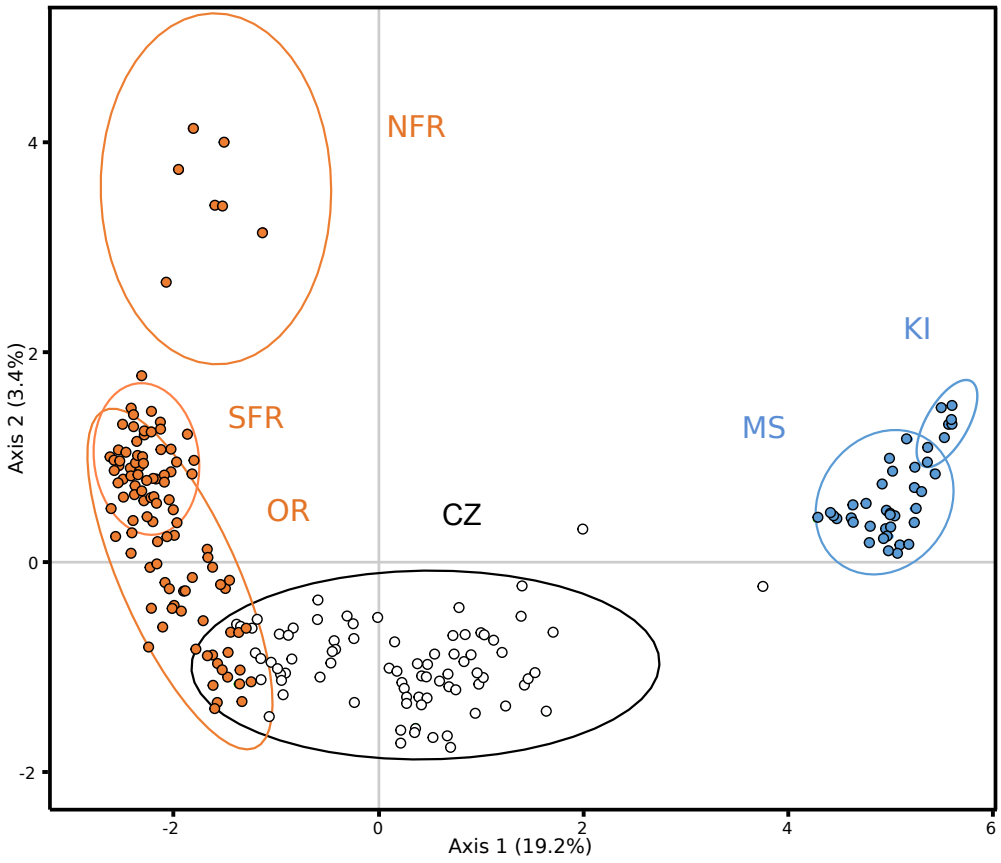


Figure 3.3. Two-dimensional principal coordinate plot (PCoA) constructed from the full SNP data set (1,333 loci) showing pairwise genetic distances between individuals. 95% confidence ellipses of each population are shown (Northern Flinders Ranges [NFR], Southern Flinders Ranges [SFR], Olary Ranges [OR], Contact Zone [CZ], Mainland South [MS], and Kangaroo Island [KI]). Two outlying admixed individuals (from the 95% confidence ellipse) were those found in the southern geographic range (MS).

The first axis of the PCoA constructed from 1,333 loci clustered all populations into two major groups corresponding to the northern and southern lineage (variance explained = 19.4%; Figure 3.3). We found evidence for the presence of hybrids along this axis, with hybrids clustered more closely to northern lineage Olary Ranges (OR) population. Population pairwise measures of F_{ST} between the contact zone and northern lineage OR/SFR/NFR populations (F_{ST} range: 0.058–0.216; Appendix 3.4) were lower relative to measures of F_{ST} between the contact zone and southern lineage MS/KI populations (F_{ST}

range: 0.203–0.293). The contact zone had the lowest inbreeding coefficient (F = 0.095) and the highest level of heterozygosity (H_{exp} = 0.084, H_{obs} = 0.079) relative to parental populations, with very similar allelic number and richness to the OR population (Table 3.1). The second PCoA axis (variance explained = 3.5%) showed population structure within each lineage, with the Northern Flinders Ranges (NFR) and Kangaroo Island (KI) populations showing differentiation from other populations of their respective lineages, and from each other (F_{ST} = 0.571). This can likely be attributed to the fact that each represents the northernmost and southernmost populations of their lineages, and KI is an insular population.

Table 3.1. Populations sampled across the species range (Northern Flinders Ranges [NFR], Southern Flinders Ranges [SFR], Olary Ranges [OR], Contact Zone [CZ], Mainland South [MS], and Kangaroo Island [KI]) with details of sample size for the SNP dataset (N), expected and observed heterozygosity (H_{exp}, H_{obs}), inbreeding coefficient (F), allele number (AN), and allelic richness (AR).

Population	N (♂ / ♀ / NA)	H_{exp}	H_{obs}	F	AN	AR
NFR	7 (2 / 0 / 5)	0.064	0.055	0.177	1629	0.222
SFR	41 (31 / 8 / 2)	0.076	0.070	0.129	2091	0.569
OR	60 (45 / 7 / 8)	0.072	0.060	0.177	2138	0.604
CZ	82 (69 / 13 / 0)	0.084	0.079	0.095	2134	0.601
MS	33 (26 / 4 / 3)	0.051	0.043	0.175	1714	0.286
KI	7 (6 / 1 / 0)	0.023	0.019	0.165	1429	0.072
Total	230	-	-	-	-	-

†Sex was unknown for a portion of loaned museum tissue samples.

3.4.2 Hybrid classification

We found a strong bias in genetic introgression with admixed individuals being predominantly backcrossed with the northern lineage. Of individuals within the contact zone, 35% had intermediate *q*-values ($0.4 < q < 0.6$) and a large proportion (65%) had q-values between 0.39 and 0.10 suggestive of backcrossing. NewHybrids analysis classified all individuals of the northern and southern lineages ($0.1 > q > 0.9$) as pure parental forms (PP ≥ 99.1%). Further, 59 of 79 individuals in the contact zone were classified to one of the six classes with a posterior probability (PP) of ≥ 95%. Additionally, 6 individuals were classified as pure northern (P1), 36 as F2 hybrids, and 19 as backcrosses to the northern lineage (B1). We did not recover any F1 hybrids or backcrosses to the southern lineage, posterior probabilities for these two categories were uniformly low (average PP = 0.0%). The two admixed individuals found in the geographic range of the southern lineage were ambiguously classified as an F2 (PP = 54.2%) and a backcross to the southern lineage (PP = 80.8%). The 22 individuals unable to be confidently classified (PP ≤ 95%) likely represent the continuum present in wild populations, unsuited for discrete classification, and difficult to distinguish even with many diagnostic markers (Boecklen and Howard 1997; Vähä and Primmer 2006).

3.4.3 Hybrid throat and dorsolateral phenotypes

The throat phenotype of wild-caught hybrid males was more similar to that of the northern lineage, exhibiting all four northern throat colour morphs with uniformly low levels of UV reflectance. Linear discriminant analyses (LDA) on throat variables showed the northern and southern lineages strongly differentiated along LD1 which was driven primarily by the proportion of blue and secondarily by QC_{UVS} and the proportion of orange (variance explained = 99.8%; Figure 3.4a, Appendix 3.5). The 95% confidence ellipses drawn for the throat phenotypes of northern and hybrid males overlie each other. The large majority of

hybrid throats cluster with northern throats. Blue coloration was not detected on the throats of pure northern males. A degree of blue coloration (≥ 5% blue pixels in segmentation analysis), however, was found on a portion of hybrid throats of each morph: grey (47.4%, N = 19), orange (5.2%, N = 39), orange-yellow (6.7%, N = 15), yellow (11.8%, N = 17; Figure 3.5a). The throat phenotype of the two hybrid individuals found in the range of the southern lineage had larger proportions of blue coloration on the throat relative to other hybrids.

In contrast to wild-caught hybrid throats, captive-bred F1 hybrid throat phenotype was more intermediate and exhibited a wide range of UV reflectance levels varying from those characteristic of the northern to southern lineages. Linear discriminant analysis on throat variables of captive-bred F1 hybrids showed strong differentiation between lineages along LD1 driven primarily by QC_{UVS} and secondarily by the proportions of blue and yellow (variance explained = 88.0%; Figure 3.4b, Appendix 3.5). The 95% confidence ellipse drawn for hybrid throats overlaps those of both northern and southern throats to a similar extent. Results were essentially unchanged when considering hybrids sired by a northern or southern male and by offspring sex. Similar to wild hybrids, all four northern throat colour morphs were recovered in captive-bred hybrids, and all but the orange-yellow morph had a proportion of individuals with a blue component: grey (32.3%, N = 31), orange (18.5%, N = 27), orange-yellow (0%, N = 2), yellow (8.3%, N = 12; Figure 3.5b). Although the artificial elevation of testosterone levels has been shown to induce the expression of northern throat colour morphs in juveniles and females (Rankin and Stuart-Fox 2015; Rankin et al. 2016), we found that it did not comparably induce the expression of the blue throat colour morph in all pure southern offspring. Therefore, the presence of blue coloration on F1 hybrid offspring throats may have been underestimated.

Dorsolateral phenotype in both wild-caught and captive-bred hybrids is intermediate between the lineages (Figures 3.4c–d). Within the contact zone, differentiation between lineages along LD1 is driven primarily by the number of breaks in the dorsolateral stripe and proportion of orange (Appendix 3.5). The 95% confidence ellipses drawn for the dorsolateral phenotypes of hybrids and northern males overlap incompletely, likely reflecting the high frequencies of backcrossing to the northern lineage. In captive-bred hybrids, comparable results were found with differentiation driven by breaks in the lateral stripe (Appendix 3.5) and the 95% confidence ellipse for hybrid dorsolateral phenotype overlaps those of both northern and southern dorsolateral phenotypes.

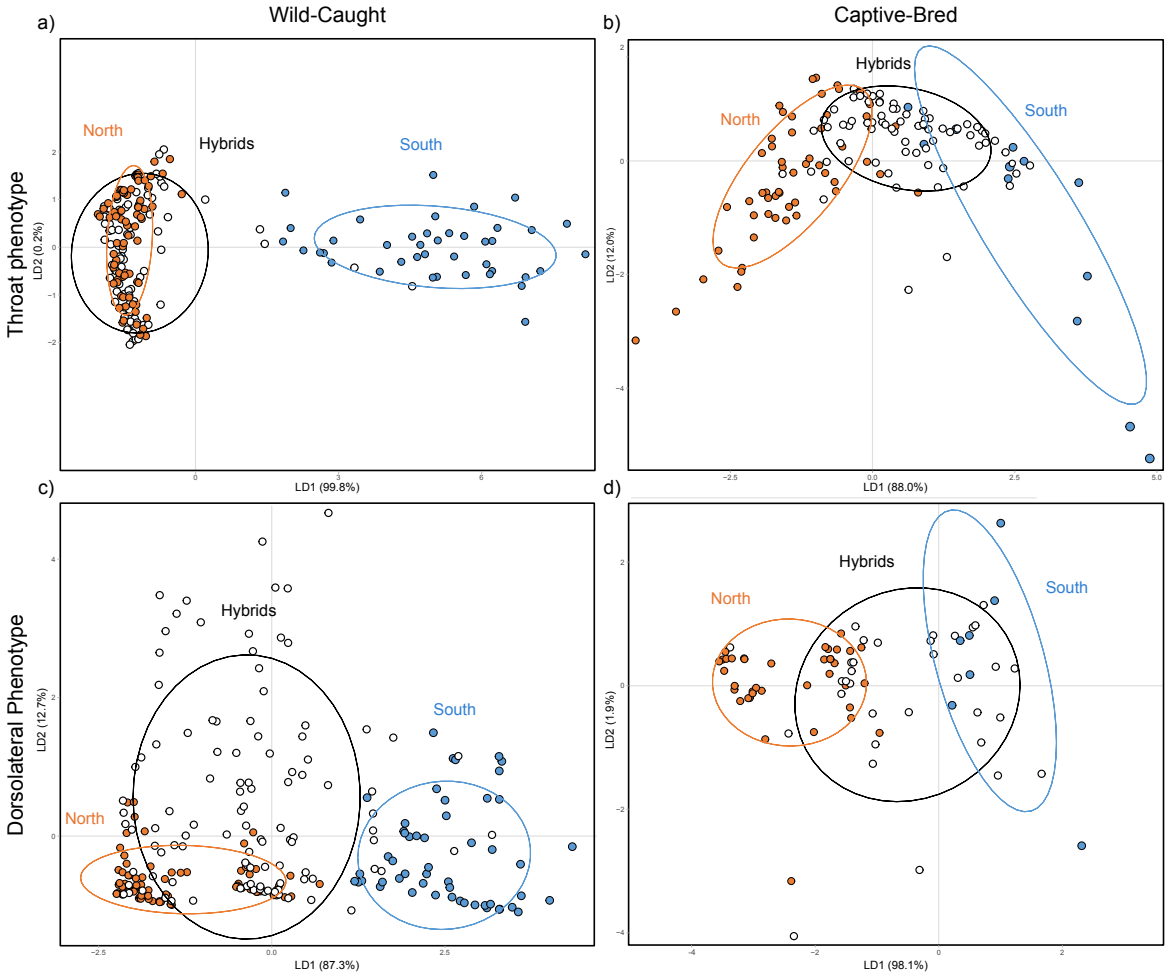


Figure 3.4. Biplots of linear discriminant analyses on (a, b) throat and (c, d) dorsolateral phenotype variables for (a, c) wild-caught individuals along the contact zone transect and (b, d) captive-bred pure and F1 hybrid offspring. 95% confidence ellipses are shown.

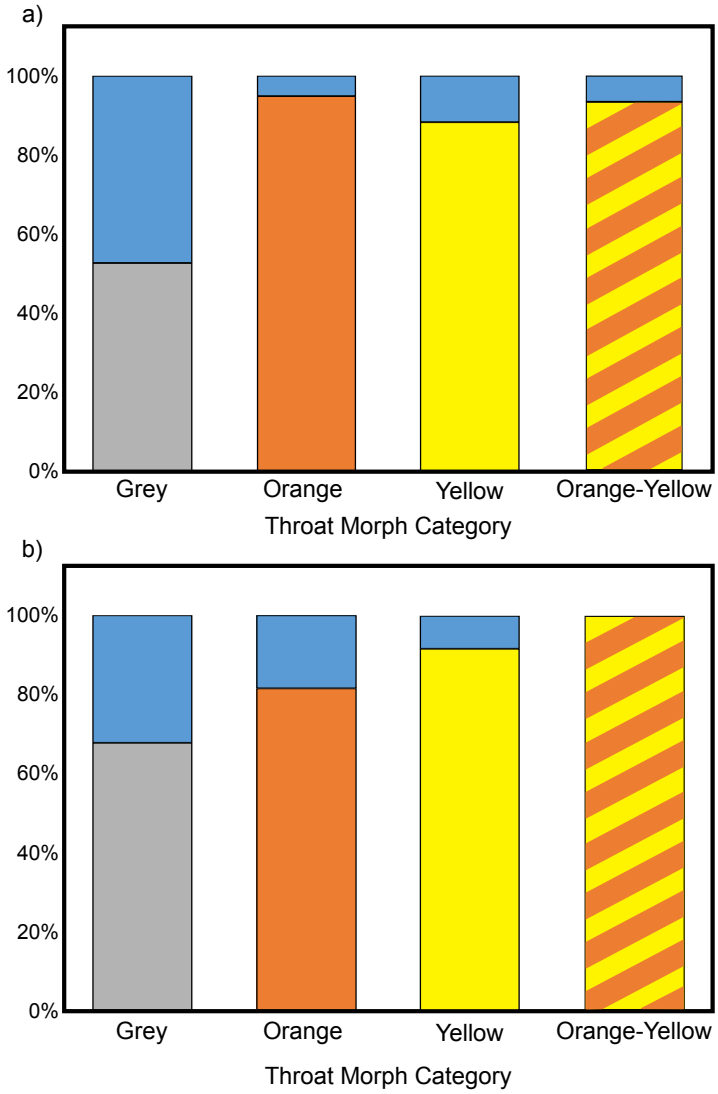


Figure 3.5. Percentage of individuals in each throat morph category which exhibit a degree of blue coloration on the throat for (a) wild-caught male hybrids (grey N = 19, orange N = 39, orange-yellow N = 15, yellow N = 17) and (b) captive-bred F1 hybrids (both sexes; grey N = 31, orange N = 27, orange-yellow N = 2, yellow N = 12).

Table 3.2. Model-averaged estimates for cline centre and width based on the AIC 95% confidence set models, with *N* representing the number of models within the AIC confident set. Cline centre is the distance (km) from the southern-most sampling site and width (w) is 1/slope at the centre. Two log-likelihood unit support limits are shown as 'low' and 'high'. See Appendix 3.6 for full details of cline models.

Trait		*N*	Centre	(low–high)	Width	(low–high)
Hybrid Index (H_{Index})		10	28.41	(22.37–33.00)	29.77	(15.05–55.84)
mtDNA		7	27.78	(14.59–28.17)	0.64	(0.08–21.85)
Dorsolateral Phenotype (D_{PC1})		2	23.02	(16.33–27.61)	13.97	(0.82–29.02)
Throat Phenotype	QC_{UVS}	4	15.90	(14.25–20.48)	8.48	(0.74–17.27)
	T_{PC1}	2	15.38	(15.06–16.61)	3.22	(2.43–7.94)

3.4.4 Geographic clines

Model-averaged clines for mtDNA and throat phenotype (QC_{UVS} and T_{PC1}) exhibited relatively narrower clines with abrupt transitions suggesting selection on these traits, compared to clines for the hybrid index (H_{Index}) and dorsolateral phenotype (D_{PC1}; Table 3.2). The best-fitting clines for the hybrid index and mtDNA had coincident centres with overlapping CIs but showed discordance in slope where the mtDNA cline was significantly narrower (H_{Index}: c = 28.41 km, w = 29.77 km; mtDNA: c = 27.78 km, w = 0.64 km; Figure 3.6a–b). The cline fitted for the dorsolateral phenotype (c = 23.02 km, w = 13.97) was coincident with that of the hybrid index (Figure 3.6c). Clines for throat phenotype (QC_{UVS} and T_{PC1}) were similar in centre and width to each other (QC_{UVS}: c = 15.90 km, w = 8.48 km; T_{PC1}: c = 15.38 km, w = 3.22 km; Figure 3.6d), their centres were displaced by 12.51 and 13.03 km to the south from the hybrid index cline centre and do not have overlapping CIs with the hybrid index cline. See Appendix 3.6 for full details of cline models in the AIC 95% confidence set for each trait.

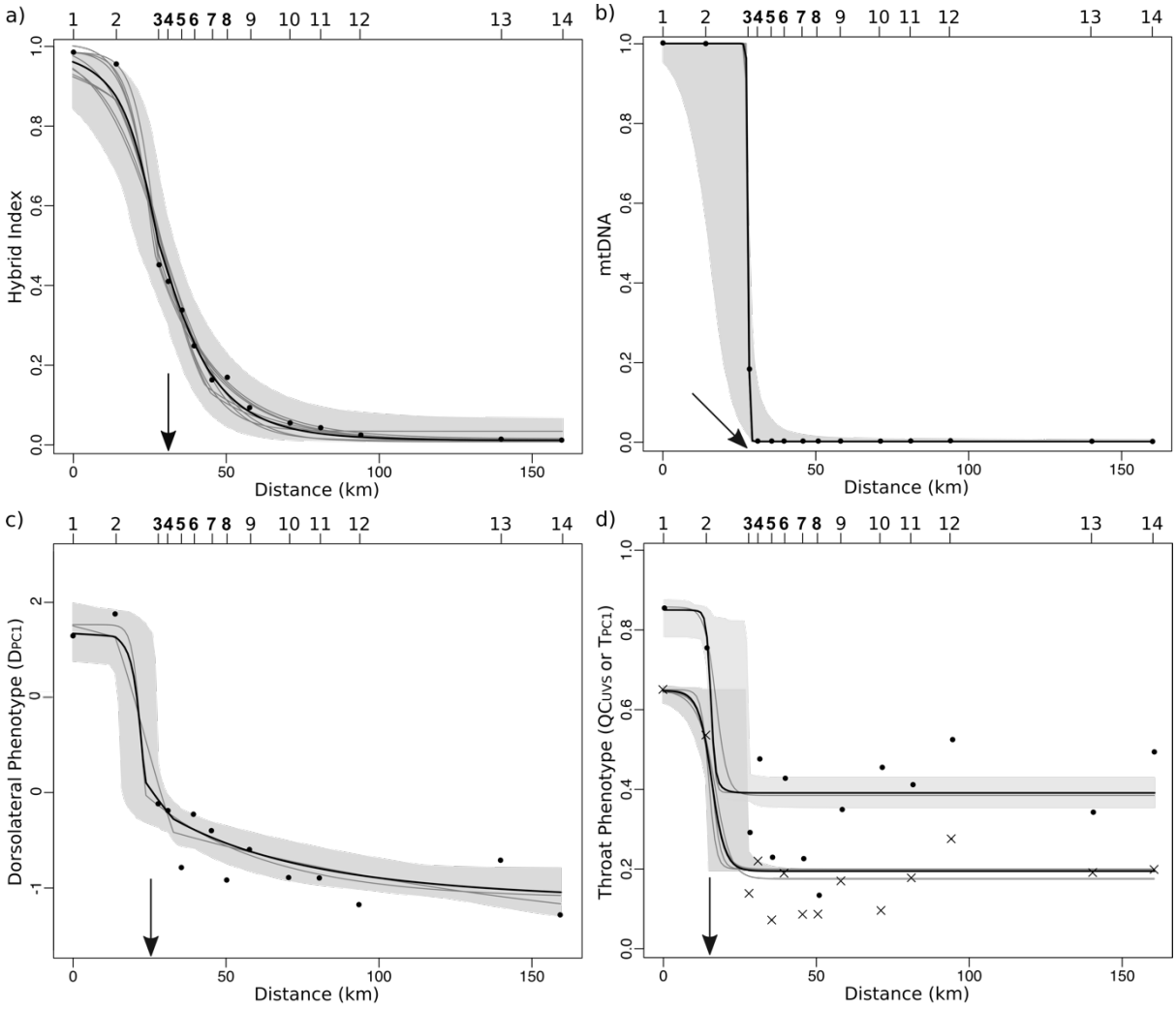


Figure 3.6. AICc model-averaged geographic clines (black) and credible regions, clines comprising the 95% confidence set of models (grey), and observed frequencies at each site for (a) the hybrid index estimated from 1333 loci (N = 154), (b) mtDNA haplotype frequencies (N = 74), (c) dorsolateral phenotype (D_{PC1}; N = 233), and (d) throat phenotype clines (N = 204 for both): quantum catch of UV receptor (QC_{UVS}; lower cline, frequencies shown with '×' symbols) and throat colouration (T_{PC1}; upper cline). Numbers along the top refer to sampling sites (see Appendix 3.2 for sample sizes at each site), bolded sites (3–8) comprise the contact zone. Transect distance is the cumulative distance from the southern-most population with increasing distance northwards. Arrows indicate cline centres; throat phenotype clines have essentially the same centres and are indicated with one arrow.

3.5 DISCUSSION

Evaluating the evolutionary outcomes of secondary contact between lineages that differ in morph composition can provide valuable insights into the role of polymorphism in speciation. We characterised genomic and phenotypic patterns across the contact zone between colour polymorphic and monomorphic lineages of *C. decresii* and concurrently measured the phenotype of captive-bred F1 hybrids. We found that the contact zone is geographically narrow relative to the parental ranges (<30 km) with no parental forms or F1 hybrids present in the genetic centre, suggesting that hybridisation is neither recent nor ongoing. Genomic introgression was asymmetric, with hybrids backcrossing almost exclusively to the northern lineage, further indicating that hybridisation between the lineages is not ongoing. This, accompanied by the prevalence of the northern lineage mtDNA haplotype across the contact zone, suggests differing levels of selection against particular recombinants. Dorsolateral phenotype across the contact zone appeared to be more associated with genetic admixture whereas there was evidence of selection for the northern throat polymorphism within the contact zone. We found all four northern throat morphs present with low UV reflectance in wild-caught hybrids, contrasting with highly variable and intermediate levels of UV reflectance in captive-bred hybrids.

The pattern of asymmetric genomic introgression, with limited backcrossing to the southern lineage, could reflect multiple evolutionary and demographic scenarios. First, extrinsic barriers may hinder dispersal and migration, in turn preventing backcrossing. The restricted gene flow to the southern lineage is associated with an approximately 12 km density trough (Figure 3.2b,c). In this region, it appears that *C. decresii* populations either occur in levels below human detection or do not currently exist despite continuous rocky habitat with no obvious barriers to dispersal (e.g. major roads, waterways, human structures; Appendix 3.9).

The density trough was surveyed thoroughly in 2016 and 2017 and briefly in 2013. The presence of suitable habitat was confirmed by surveys on foot, satellite imagery (Appendix 3.9), and/or flying drone surveys. The absence of any significant physical barriers suggests that extrinsic factors are unlikely to shape genetic patterns in the contact zone.

Second, intrinsic genomic incompatibilities could exist which limit backcrossing via reinforcement (i.e. selection against maladaptive hybridsation). The absence of extrinsic barriers coupled with the narrower and more abrupt transition exhibited by the mtDNA cline relative to the hybrid index, and the rarity of admixed individuals containing the southern mtDNA haplotype, provide evidence of selection against particular recombinants across the contact zone. Captive breeding experiments show no significant prezygotic barriers to the formation of F1 hybrids with either parental mtDNA haplotype (Dong, Rankin, McLean, & Stuart-Fox, unpublished data), although their reproductive viability is unknown. However, stronger incompatibilities and hybrid breakdown are more frequently seen in F2 and later generation hybrids during the early stages of divergence. This has been described extensively for both Dobzhansky-Muller incompatibilities (reviewed in Coyne & Orr, 2004) and for cytonuclear incompatibilities (Ellison & Burton, 2008; Fishman & Willis, 2006; Meiklejohn et al., 2013; Niehusis, Judson, & Gadau, 2008). Overall, our results support the hypothesis that intrinsic factors may limit gene flow, and backcrossing to the southern lineage, regardless of mtDNA haplotype, may result in offspring that are inviable, infertile, or suffer greatly reduced fitness.

A third possibility is that the pattern of asymmetric genomic introgression coupled with the prevalence of northern mtDNA could potentially be explained by demographic circumstances. For instance, a greater abundance of northern individuals during initial periods of hybridisation with continued asymmetric migration into the contact zone, or the

sex-biased dispersal of southern males to the north. A greater continued abundance of northern individuals in the contact zone should result in a greater proportion of northern genomic background (southern genomic signature quickly eroded by northern contribution). This is not consistent with the close to equal genomic proportions found in many hybrids at the genetic centre. Male-biased dispersal could produce population genetic structure consistent with our results, but dispersal rates and behaviour have not been examined for *C. decresii*. There is evidence of male-biased dispersal of lizards in fragmented landscapes (Levy, Kennington, Tomkins, & Lebas, 2010; Stow, Sunnucks, Birscoe, & Gardner, 2001; Sumner, 2005), and of male juvenile and male adult *Anolis sagrei* lizards (Calsbeek, 2009; Calsbeek, Duryea, Parker, & Cox, 2014). However, it is uncertain why male-biased dispersal would exclusively be observed in the southern lineage and not in the northern.

Phenotypic patterns recovered from within the contact zone are consistent with the explanation that the northern throat phenotype is selectively favoured within the contact zone, in terms of both discrete throat morphs and UV reflectance. Although throat and dorsolateral coloration are both sexually dichromatic traits, the throat phenotype clines were both displaced to the southern end of the contact zone, with the northern throat phenotype prevalent in hybrids. By contrast, the dorsolateral coloration cline was more concordant with the genetic cline. The prevalence of the northern throat phenotype (e.g. displaced cline) could theoretically arise from genetic dominance. In this scenario, captive-bred F1 hybrids would be expected to have largely northern throat phenotypes which contrasts with the observed intermediate phenotypes. Another possibility is that this phenotypic pattern is due to extensive backcrossing to the northern lineage. While some wild hybrids may have northern throat phenotypes because they are heavily backcrossed to the northern lineage, the prevalence of northern phenotype in hybrids with approximately equal proportions of

northern and southern genomic backgrounds points towards selection against the southern blue phenotype.

We did not detect the loss of any northern throat morphs across the contact zone or in F1 hybrids, indicating that co-adapted trait complexes of the northern polymorphism are stable despite genetic disruption. Although hybrid throats were objectively and confidently classified into discrete northern throat morphs, we observed a proportion of each morph which exhibited a degree of intermediate nature where an area of blue coloration was present. There was a notably higher proportion of grey morphs which had blue coloration present. This may be due to the similar mechanism by which grey and blue skin are produced in *C. decresii*, both from melanin pigments in melanophores with blue skin incorporating an additional structural component produced from guanine crystals in iridophores (McLean et al. 2017). Nevertheless, we did not detect any hybrid throats that exhibited a true southern throat phenotype with both predominantly blue throat coloration and a high level of UV reflectance. This, in addition to the wide range of UV reflectance found in captive-bred F1 hybrids, points toward the uncoupling of blue coloration and UV reflectance in hybrids driven by stronger negative sexual selection against UV reflectance as a singular trait.

Selection for the northern throat polymorphism could be driven by mating behaviour which evolved via reinforcement and/or microhabitat characteristics. Mate preference trials with captive *C. decresii* predict a greater frequency of mating between southern females with northern males than the reciprocal combination (McLean et al. 2020). This contrasts with patterns observed within the contact zone, indicating that prezygotic barriers between lineages have not evolved in allopatry. However, prezygotic barriers are expected to form in the contact zone because postzygotic barriers (inferred from low frequency of hybrids backcrossed to the south) should select for the formation of prezygotic barriers to prevent

maladaptive hybridisation (i.e. reinforcement). Extrinsic factors could also contribute to the northern throat phenotype having a competitive advantage in the contact zone, for example, due to a relatively higher conspicuousness to conspecifics. Throat coloration of each lineage is more conspicuous against the predominant background colours of native lichen in their respective ranges (McLean et al. 2014a). Within the contact zone, lichen coloration is similar to that found in the range of the northern lineage, predominantly pale green. Therefore, sexual selection may operate through male-male interactions or female choice to result in a reproductive advantage of the northern throat phenotype as a more conspicuous and detectable visual signal. Selection has been shown to drive the rapid spread of an advantageous trait/phenotype across contact zones, and in some cases into the other parental lineage (Stein and Uy 2006; Pardo-Diaz et al. 2012; Baldassarre et al. 2014; While et al. 2015). In contrast, the dorsolateral phenotype cline indicates that it is not under similarly strong selection and appears to play a lesser role in reproductive isolation in sympatry. Male dorsolateral coloration may be constrained as it is under both sexual and natural selection because of its dual function as a intraspecific signal and in minimising conspicuousness to predators against native lichen (McLean et al. 2014a; Ramos and Peters 2016).

3.6 CONCLUSIONS

In summary, we provide evidence that sexual signal divergence and intrinsic genetic incompatibilities play an important role in restricting gene flow between polymorphic and monomorphic lineages of *C. decresii*. Our results suggest that the reciprocally monophyletic lineages remain distinct in the face of gene flow. Thus, consideration should be given towards elevating the lineages to species status based on genetic and phenotypic divergence with restricted gene flow. The stability of the northern polymorphism despite genetic disruption is consistent with the theoretical genetic architecture of colour polymorphism and correlated

traits, namely control by pleiotropic or tightly linked genes. Taken together, these results lend support to the view that polymorphism can promote speciation.

3.7 ACKNOWLEDGEMENTS

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3.8 APPENDICES

Appendix 3.1. Details of all individuals used in the study, including if the individual was sequenced (SNPs and/or mitochondrial ND4 gene), had dorsal/ventral photos and spectral reflectance measurements, and the sex (M = male, F = female). Population (Southern: Kangaroo Island [KI], Mainland South [MS]; Northern: Northern Flinders Ranges [NFR], Southern Flinders Ranges [SFR], Olary Ranges [OR]; and Contact Zone [CZ]), year, and cline site (if applicable) are shown.

ID	SNPs	ND4	Photos	Specs	Sex	Pop	Year	Cline Site	Latitude	Longitude
KI206	Y	N	N	N	M	KI	2010	NA	-35.965	136.653
KI208	Y	N	N	N	M	KI	2011	NA	-35.965	136.653
KI194	Y	N	N	N	F	KI	2010	NA	-35.964	136.653
KI179	Y	N	N	N	M	KI	2010	NA	-35.963	136.654
KI193	Y	N	N	N	M	KI	2010	NA	-35.963	136.654
KI181	Y	N	N	N	M	KI	2010	NA	-35.960	136.655
KI215	Y	N	N	N	M	KI	2011	NA	-35.621	137.210
R45184	Y	N	N	N	NA	MS	1994	NA	-35.642	138.329
R49234	Y	N	N	N	NA	MS	1996	NA	-35.601	138.279
R53785	Y	N	N	N	F	MS	2000	NA	-35.194	138.919
R55041	Y	N	N	N	F	MS	2000	NA	-35.160	138.578
M232	Y	N	N	N	M	MS	2011	NA	-34.908	138.707
M235	Y	N	N	N	M	MS	2011	NA	-34.906	138.702
M248	Y	N	N	N	M	MS	2011	NA	-34.906	138.707
R42978	Y	N	N	N	NA	MS	1993	NA	-34.683	138.850
631	Y	N	N	N	M	MS	2012	NA	-34.582	139.015
KS630	Y	N	N	N	M	MS	2012	NA	-34.582	139.015
KS631	Y	N	N	N	M	MS	2012	NA	-34.582	139.015
KS632	Y	N	N	N	M	MS	2012	NA	-34.582	139.015
KS634	Y	N	N	N	F	MS	2012	NA	-34.581	139.015
R27416	N	Y	N	N	NA	MS	2012	1	-34.842	139.133

638	N	Y	N	N	F	MS	2012	1	-34.831	139.130
639	N	Y	N	N	F	MS	2012	1	-34.494	139.201
640	N	Y	N	N	F	MS	2012	1	-34.830	139.129
641	N	Y	N	N	M	MS	2012	1	-34.494	139.201
642	N	Y	N	N	F	MS	2012	1	-34.833	139.128
PB1	Y	N	Y	N	M	MS	2013	1	-34.834	139.128
S42	Y	N	Y	Y	M	MS	2016	1	-34.830	139.129
S32	N	N	Y	Y	M	MS	2015	1	-34.830	139.128
S44	N	N	Y	Y	M	MS	2016	1	-34.829	139.129
S31	Y	Y	Y	Y	M	MS	2015	1	-34.829	139.130
S39	N	N	Y	Y	M	MS	2015	1	-34.829	139.129
S47	Y	Y	Y	Y	M	MS	2016	1	-34.828	139.124
S30	N	N	Y	Y	M	MS	2015	1	-34.826	139.129
S40	N	N	Y	Y	M	MS	2015	1	-34.826	139.160
S33	N	N	Y	Y	M	MS	2015	1	-34.822	139.162
S29	Y	N	Y	Y	M	MS	2015	1	-34.822	139.116
S8	Y	Y	N	N	F	MS	2015	1	-34.822	139.116
S27	N	N	Y	Y	M	MS	2015	1	-34.821	139.121
S28	N	N	Y	Y	M	MS	2015	1	-34.821	139.121
PB9	N	N	Y	N	M	MS	2013	1	-34.821	139.160
PB10	N	N	Y	N	M	MS	2013	1	-34.821	139.160
PB4	N	N	Y	N	M	MS	2013	1	-34.820	139.137
PB8	Y	Y	Y	N	M	MS	2013	1	-34.821	139.160
641	N	N	Y	Y	M	MS	2012	1	-34.494	139.201
PB2	N	N	Y	N	M	MS	2013	1	-34.820	139.160
PB3	N	N	Y	N	M	MS	2013	1	-34.820	139.132
PB5	N	N	Y	N	M	MS	2013	1	-34.819	139.162
S21	Y	N	Y	Y	M	MS	2015	1	-34.818	139.162
PB6	N	N	Y	N	M	MS	2013	1	-34.818	139.163
S36	N	N	Y	Y	M	MS	2015	1	-34.818	139.163
PB7	N	N	Y	N	M	MS	2013	1	-34.818	139.164

S26	N	N	Y	Y	M	MS	2015	1	-34.817	139.117
710	Y	N	N	N	M	MS	2013	1	-34.817	139.117
S25	Y	N	Y	Y	M	MS	2015	1	-34.817	139.116
S24	N	N	Y	Y	M	MS	2015	1	-34.817	139.164
S35	N	N	Y	Y	M	MS	2015	1	-34.817	139.114
S22	N	N	Y	Y	M	MS	2015	1	-34.817	139.165
S23	N	N	Y	Y	M	MS	2015	1	-34.817	139.165
S37	N	N	Y	Y	M	MS	2015	1	-34.816	139.163
S38	N	N	Y	Y	M	MS	2015	1	-33.022	138.000
1142	N	N	Y	Y	M	MS	2017	2	-34.793	139.203
1179	N	N	Y	Y	M	MS	2017	2	-34.742	139.212
1177	Y	N	Y	Y	M	MS	2017	2	-34.742	139.211
1178	N	N	Y	Y	M	MS	2017	2	-34.740	139.211
1145	Y	N	Y	Y	M	MS	2017	2	-34.726	139.214
1146	Y	N	Y	Y	M	MS	2017	2	-34.725	139.214
703	Y	N	Y	N	M	MS	2013	2	-34.725	139.214
1140	N	N	Y	Y	M	MS	2017	2	-34.723	139.216
1143	N	N	Y	Y	M	MS	2017	2	-34.723	139.214
1141	Y	N	Y	Y	M	MS	2017	2	-34.723	139.215
1144	Y	N	Y	Y	M	MS	2017	2	-34.723	139.215
1171	N	N	Y	Y	M	MS	2017	2	-34.721	139.215
1164	N	N	Y	Y	M	MS	2017	2	-34.721	139.215
1167	N	N	Y	Y	M	MS	2017	2	-34.721	139.213
1168	Y	N	Y	Y	M	MS	2017	2	-34.721	139.213
1169	N	N	Y	Y	M	MS	2017	2	-34.721	139.213
1170	N	N	Y	Y	M	MS	2017	2	-34.721	139.213
1166	N	N	Y	Y	M	MS	2017	2	-34.720	139.213
709	Y	Y	N	N	M	MS	2013	2	-34.720	139.207
700	N	N	Y	N	M	MS	2013	2	-34.720	139.214
1165	Y	N	Y	Y	M	MS	2017	2	-34.720	139.214
706	Y	N	N	N	M	MS	2013	2	-34.718	139.206

S1107	Y	N	Y	Y	M	MS	2016	2	-34.701	139.206
1180	N	N	Y	Y	M	CZ	2017	3	-34.591	139.218
H1106	Y	N	Y	Y	M	CZ	2016	3	-34.590	139.218
1186	N	N	Y	Y	M	CZ	2017	3	-34.590	139.209
1185	Y	Y	Y	Y	M	CZ	2017	3	-34.589	139.211
1187	N	N	Y	Y	M	CZ	2017	3	-34.588	139.215
1184	N	N	Y	Y	M	CZ	2017	3	-34.588	139.215
H1104	Y	Y	N	N	F	CZ	2016	3	-34.588	139.218
1182	N	N	Y	Y	M	CZ	2017	3	-34.588	139.217
1183	N	N	Y	Y	M	CZ	2017	3	-34.587	139.216
759	Y	N	Y	N	M	CZ	2013	3	-34.587	139.207
758	N	N	Y	N	M	CZ	2013	3	-34.587	139.206
756	Y	Y	Y	N	M	CZ	2013	3	-34.586	139.206
754	Y	N	N	N	M	CZ	2013	3	-34.586	139.205
1215	N	N	Y	Y	M	CZ	2017	3	-34.585	139.216
1217	N	N	Y	Y	M	CZ	2017	3	-34.585	139.216
1216	N	N	Y	Y	M	CZ	2017	3	-34.585	139.216
H1100	Y	N	Y	Y	M	CZ	2016	3	-34.583	139.215
H1101	Y	N	Y	Y	M	CZ	2016	3	-34.583	139.215
H1102	Y	Y	N	N	F	CZ	2016	3	-34.579	139.215
H1103	Y	Y	N	N	F	CZ	2016	3	-34.577	139.215
H1099	Y	N	Y	Y	M	CZ	2016	3	-34.577	139.203
H1021	Y	N	Y	Y	M	CZ	2015	4	-34.571	139.205
739	Y	N	Y	N	M	CZ	2013	4	-34.564	139.211
738	Y	Y	N	N	F	CZ	2013	4	-34.563	139.208
740	Y	N	Y	N	M	CZ	2013	4	-34.563	139.213
741	Y	Y	N	N	M	CZ	2013	4	-34.563	139.212
737	Y	N	Y	N	M	CZ	2013	4	-34.563	139.207
753	N	N	Y	N	M	CZ	2013	4	-34.561	139.193
716	Y	N	Y	N	M	CZ	2013	4	-34.561	139.202
712	N	N	Y	N	M	CZ	2013	4	-34.561	139.203

713	Y	N	N	N	M	CZ	2013	4	-34.559	139.205
H1020	Y	N	Y	Y	M	CZ	2015	4	-34.558	139.202
H1019	N	N	Y	Y	M	CZ	2015	4	-34.558	139.203
H1009	N	N	Y	Y	M	CZ	2015	4	-34.558	139.177
H1018	N	N	Y	Y	M	CZ	2015	4	-34.557	139.204
715	N	N	Y	N	M	CZ	2013	4	-34.557	139.204
H1017	Y	Y	Y	Y	M	CZ	2015	4	-34.557	139.205
H1051	Y	N	Y	Y	M	CZ	2016	4	-34.557	139.200
H1039	N	N	Y	Y	M	CZ	2015	4	-34.556	139.199
H1007	Y	N	Y	Y	M	CZ	2015	4	-34.556	139.200
H1048	N	N	Y	Y	M	CZ	2015	4	-34.556	139.199
H1008	Y	N	Y	Y	M	CZ	2015	4	-34.556	139.198
H1046	N	N	Y	Y	M	CZ	2015	4	-34.556	139.198
746	N	N	Y	N	M	CZ	2013	4	-34.556	139.199
742	Y	Y	N	N	F	CZ	2013	4	-34.556	139.198
H1049	Y	N	Y	Y	M	CZ	2016	4	-34.556	139.199
743	N	N	Y	N	M	CZ	2013	4	-34.556	139.198
745	N	N	Y	N	M	CZ	2013	4	-34.556	139.197
H1045	N	N	Y	Y	M	CZ	2015	4	-34.556	139.199
H1052	Y	N	N	N	F	CZ	2016	4	-34.556	139.200
H1050	Y	N	Y	Y	M	CZ	2016	4	-34.556	139.200
H1044	N	N	Y	Y	M	CZ	2015	4	-34.556	139.200
H1055	Y	Y	N	N	F	CZ	2016	4	-34.556	139.201
744	N	N	Y	N	M	CZ	2013	4	-34.556	139.202
H1053	Y	Y	Y	Y	M	CZ	2016	4	-34.556	139.200
H1054	Y	N	Y	Y	M	CZ	2016	4	-34.556	139.202
H1056	Y	Y	N	N	F	CZ	2016	4	-34.556	139.202
H1010	N	N	Y	Y	M	CZ	2015	4	-34.556	139.203
H1014	Y	N	Y	Y	M	CZ	2015	4	-34.556	139.203
H1016	Y	Y	Y	Y	M	CZ	2015	4	-34.556	139.202
H1022	Y	Y	Y	Y	M	CZ	2015	4	-34.556	139.201

748	N	N	Y	N	M	CZ	2013	4	-34.556	139.199
H1011	Y	N	Y	Y	M	CZ	2015	4	-34.555	139.203
H1015	N	N	Y	Y	M	CZ	2015	4	-34.555	137.203
H1013	Y	N	Y	Y	M	CZ	2015	4	-34.555	139.203
H1012	Y	N	Y	Y	M	CZ	2015	4	-34.555	139.203
H1023	N	N	Y	Y	M	CZ	2015	4	-34.555	139.202
H1027	Y	Y	Y	Y	M	CZ	2015	4	-34.555	139.206
H1041	N	N	Y	Y	M	CZ	2015	4	-34.555	139.205
750	N	N	Y	N	M	CZ	2013	4	-34.555	139.202
H1042	N	N	Y	Y	M	CZ	2015	4	-34.555	139.204
H1043	N	N	Y	Y	M	CZ	2015	4	-34.555	139.204
H1040	N	N	Y	Y	M	CZ	2015	4	-34.555	139.204
H1029	N	N	Y	Y	M	CZ	2015	4	-34.555	139.204
H1025	Y	N	Y	Y	M	CZ	2015	4	-34.555	139.207
H1026	N	N	Y	Y	M	CZ	2015	4	-34.554	139.207
H1024	N	N	Y	Y	M	CZ	2015	4	-34.554	139.202
H1028	N	N	Y	Y	M	CZ	2015	4	-34.554	139.206
751	N	N	Y	N	M	CZ	2013	4	-34.554	139.202
749	Y	Y	N	N	F	CZ	2013	4	-34.552	139.203
H1086	Y	N	Y	Y	M	CZ	2016	4	-34.551	139.192
H1089	Y	N	Y	Y	M	CZ	2016	5	-34.542	139.197
H1091	Y	Y	Y	Y	M	CZ	2016	5	-34.540	139.202
H1092	Y	Y	Y	Y	M	CZ	2016	5	-34.540	139.202
H1057	Y	N	Y	Y	M	CZ	2016	5	-34.540	139.198
H1059	Y	Y	Y	Y	M	CZ	2016	5	-34.539	139.196
H1090	Y	N	Y	Y	M	CZ	2016	5	-34.539	139.194
636	Y	N	N	N	M	CZ	2012	5	-34.537	138.999
H1097	Y	Y	Y	Y	M	CZ	2016	5	-34.534	139.198
H1060	Y	Y	Y	Y	M	CZ	2016	5	-34.531	139.206
H1093	N	N	Y	Y	M	CZ	2016	5	-34.528	139.197
H1096	Y	N	Y	Y	M	CZ	2016	5	-34.526	139.195

H1098	Y	Y	Y	Y	M	CZ	2016	5	-34.526	139.195
637	N	Y	N	N	F	CZ	2012	6	-34.494	139.201
H1084	Y	N	Y	Y	M	CZ	2016	6	-34.515	139.200
H1075	Y	N	N	N	F	CZ	2016	6	-34.513	139.201
H1076	N	N	Y	Y	M	CZ	2016	6	-34.513	139.201
H1082	Y	Y	Y	Y	M	CZ	2016	6	-34.513	139.200
H1078	Y	N	Y	Y	M	CZ	2016	6	-34.513	139.200
H1079	Y	N	Y	Y	M	CZ	2016	6	-34.508	139.202
H1081	Y	N	Y	Y	M	CZ	2016	6	-34.507	139.202
H1072	N	N	Y	Y	M	CZ	2016	6	-34.503	139.205
H1074	Y	N	Y	Y	M	CZ	2016	6	-34.503	139.205
H1071	Y	Y	Y	Y	M	CZ	2016	6	-34.498	139.201
H1068	Y	N	Y	Y	M	CZ	2016	6	-34.495	139.202
H1069	N	N	Y	Y	M	CZ	2016	6	-34.495	139.202
H1064	Y	N	Y	Y	M	CZ	2016	6	-34.494	139.201
H1065	N	N	Y	Y	M	CZ	2016	6	-34.494	139.202
1218	N	N	Y	Y	M	CZ	2017	6	-34.493	139.202
1220	Y	N	Y	Y	M	CZ	2017	6	-34.492	139.203
H1067	Y	N	Y	Y	M	CZ	2016	6	-34.490	139.203
1222	N	N	Y	Y	M	CZ	2017	6	-34.494	139.200
1221	N	N	Y	Y	M	CZ	2017	6	-34.494	139.201
1223	Y	N	Y	Y	M	CZ	2017	7	34.494	139.201
H1108	Y	N	N	N	M	CZ	2016	7	-34.465	139.203
H1109	Y	N	Y	Y	M	CZ	2016	7	-34.464	139.203
H1111	Y	Y	Y	Y	M	CZ	2016	7	-34.451	139.218
H1110	Y	N	N	N	F	CZ	2016	7	-34.451	139.219
H1113	Y	N	Y	Y	M	CZ	2016	7	-34.449	139.215
H1112	Y	N	Y	Y	M	CZ	2016	7	-34.448	139.209
H1114	Y	N	Y	Y	M	CZ	2016	7	-34.443	39.210
H1115	Y	N	N	N	F	CZ	2016	7	-34.436	139.212
627	N	Y	N	N	M	CZ	2012	8	-34.411	139.159

628	N	Y	N	N	F	CZ	2012	8	-34.411	139.159
629	N	Y	N	N	F	CZ	2012	8	-34.413	139.161
719	Y	Y	N	N	M	CZ	2013	8	-34.421	139.187
721	Y	N	N	N	M	CZ	2013	8	-34.414	139.168
728	Y	N	N	N	M	CZ	2013	8	-34.412	139.164
720	Y	N	N	N	M	CZ	2013	8	-34.411	139.158
H1139	Y	N	Y	Y	M	CZ	2016	8	-34.409	139.210
H1136	Y	N	N	N	F	CZ	2016	8	-34.408	139.215
H1137	Y	N	Y	Y	M	CZ	2016	8	-34.408	139.215
H1117	Y	N	Y	Y	M	OR	2016	9	-34.366	139.164
H1116	Y	Y	Y	Y	M	OR	2016	9	-34.364	139.160
1173	Y	N	Y	Y	M	OR	2017	9	-34.360	139.208
1174	N	N	Y	Y	M	OR	2017	9	-34.360	139.208
1175	Y	N	Y	Y	M	OR	2017	9	-34.358	139.209
1210	N	N	Y	Y	M	OR	2017	9	-34.351	139.190
1224	Y	N	Y	Y	M	OR	2017	9	-34.350	139.188
1225	N	N	Y	Y	M	OR	2017	9	-34.350	139.188
1211	N	N	Y	Y	M	OR	2017	9	-34.350	139.188
1209	N	N	Y	Y	M	OR	2017	9	-34.349	139.189
1208	Y	N	Y	Y	M	OR	2017	9	-34.349	139.188
1212	Y	Y	Y	Y	M	OR	2017	9	-34.348	139.189
1214	Y	N	Y	Y	M	OR	2017	9	-34.348	139.189
1213	Y	N	Y	Y	M	OR	2017	9	-34.348	139.189
1159	N	N	Y	Y	M	OR	2017	9	-34.332	139.173
1176	N	N	Y	Y	M	OR	2017	9	-34.332	139.172
1163	Y	N	Y	Y	M	OR	2017	9	-34.332	139.170
1162	Y	Y	Y	Y	M	OR	2017	9	-34.331	139.173
1160	Y	N	Y	Y	M	OR	2017	9	-34.331	139.174
1161	Y	Y	N	N	F	OR	2017	9	-34.331	139.174
H1121	N	N	Y	Y	M	OR	2016	9	-34.331	139.174
1158	Y	N	Y	Y	M	OR	2017	9	-34.331	139.174

H1122	Y	N	Y	Y	M	OR	2016	9	-34.331	139.173
H1123	Y	Y	Y	Y	M	OR	2016	9	-34.331	139.174
H1124	Y	N	Y	Y	M	OR	2016	10	-34.231	139.118
H1125	Y	Y	Y	Y	M	OR	2016	10	-34.228	139.118
1207	Y	N	Y	Y	M	OR	2017	11	-34.167	139.069
H1133	Y	N	Y	Y	M	OR	2016	11	-34.167	139.069
1206	N	N	Y	Y	M	OR	2017	11	-34.167	139.069
1156	Y	N	Y	Y	M	OR	2017	11	-34.166	139.070
1155	Y	N	Y	Y	M	OR	2017	11	-34.166	139.071
1152	Y	Y	Y	Y	M	OR	2017	11	-34.132	139.048
1148	Y	N	Y	Y	M	OR	2017	11	-34.128	139.048
1154	Y	N	Y	Y	M	OR	2017	11	-34.127	139.048
1149	Y	Y	N	N	F	OR	2017	11	-34.127	139.048
1151	N	N	Y	Y	M	OR	2017	11	-34.127	139.048
H1131	Y	Y	Y	Y	M	OR	2016	11	-34.127	139.049
1153	N	N	Y	Y	M	OR	2017	11	-34.125	139.047
H1135	Y	N	Y	Y	M	OR	2016	12	-34.091	139.035
626	Y	Y	N	N	M	OR	2012	12	-34.070	138.957
R58318	Y	Y	N	N	NA	OR	2003	12	-34.031	138.935
N27	Y	N	Y	Y	M	OR	2015	12	-33.935	138.952
N16	Y	N	Y	Y	M	OR	2015	12	-33.934	138.952
623	Y	N	N	N	M	OR	2012	12	-33.934	138.952
618	Y	Y	N	N	M	OR	2012	12	-33.934	138.952
R26108	Y	N	N	N	NA	OR	1984	12	-33.917	138.550
619	N	Y	N	N	M	OR	2012	12	-33.934	138.952
620	N	Y	N	N	F	OR	2012	12	-33.934	138.952
622	N	Y	N	N	F	OR	2012	12	-33.934	138.952
624	N	Y	N	N	F	OR	2012	12	-	-
625	N	Y	N	N	F	OR	2012	12	-34.070	138.957
N28	N	N	Y	Y	M	OR	2015	12	-33.934	138.953
623	Y	N	N	N	NA	OR	1992	12	-33.934	138.952

R40708	Y	Y	N	N	NA	OR	1992	13	-33.667	138.950
1205	N	N	Y	Y	M	OR	2017	13	-33.662	139.042
1197	N	N	Y	Y	M	OR	2017	13	-33.631	139.004
1195	Y	Y	Y	Y	M	OR	2017	13	-33.631	139.004
1203	N	N	Y	Y	M	OR	2017	13	-33.628	138.996
1194	N	N	Y	Y	M	OR	2017	13	-33.628	139.003
1190	N	Y	Y	Y	M	OR	2017	13	-33.628	139.001
1189	Y	Y	Y	Y	M	OR	2017	13	-33.628	138.999
1193	N	N	Y	Y	M	OR	2017	13	-33.628	139.002
1188	N	N	Y	Y	M	OR	2017	13	-33.627	138.999
1191	Y	N	Y	Y	M	OR	2017	13	-33.627	138.994
1200	Y	Y	Y	Y	M	OR	2017	13	-33.615	138.996
1201	N	N	Y	Y	M	OR	2017	13	-33.615	138.996
1202	N	N	Y	Y	M	OR	2017	13	-33.615	138.996
R41631	N	Y	N	N	M	OR	1992	13	-33.675	138.961
R38012	N	Y	N	N	NA	OR	1991	13	-33.833	139.017
91	N	Y	N	N	M	OR	2010	13	-33.835	139.034
N32	N	N	Y	Y	M	OR	2015	14	-33.444	139.102
N36	Y	N	Y	Y	M	OR	2015	14	-33.442	139.104
N34	N	N	Y	Y	M	OR	2015	14	-33.442	139.104
N30	Y	Y	Y	Y	M	OR	2015	14	-33.441	139.103
N33	N	N	Y	Y	M	OR	2015	14	-33.441	139.103
N42	N	N	Y	Y	M	OR	2016	14	-33.439	139.103
N49	N	N	Y	Y	M	OR	2016	14	-33.439	139.101
N44	Y	Y	N	N	F	OR	2016	14	-33.439	139.101
N21	Y	N	Y	Y	M	OR	2015	14	-33.411	139.111
N40	Y	Y	Y	Y	M	OR	2015	14	-33.411	139.111
N25	Y	Y	Y	Y	M	OR	2015	14	-33.410	139.095
N24	N	N	Y	Y	M	OR	2015	14	-33.410	139.096
N29	N	N	Y	Y	M	OR	2015	14	-33.411	139.112
N39	N	N	Y	Y	M	OR	2015	14	-33.411	139.111

N26	N	N	Y	Y	M	OR	2015	14	-33.411	139.102
N22	N	N	Y	Y	M	OR	2015	14	-33.411	139.103
N23	N	N	Y	Y	M	OR	2015	14	-33.412	139.098
N37	N	N	Y	Y	M	OR	2015	14	-33.442	139.101
N31	N	N	Y	Y	M	OR	2015	14	-33.442	139.107
N38	N	N	Y	Y	M	OR	2015	14	-33.444	139.103
R58759	N	Y	N	N	M	OR	2004	14	-33.434	139.079
R53529	N	Y	N	N	NA	OR	1999	14	-33.345	139.261
R41362	Y	N	N	N	NA	OR	1992	NA	-33.350	139.103
R56417	Y	N	N	N	NA	OR	2001	NA	-33.234	138.250
R41167	Y	N	N	N	M	OR	1992	NA	-33.200	139.619
R41638	Y	N	N	N	F	OR	1992	NA	-33.124	139.196
R41771	Y	N	N	N	NA	OR	1992	NA	-33.061	139.831
BS377	Y	N	N	N	F	OR	2011	NA	-32.095	140.274
BS378	Y	N	N	N	M	OR	2011	NA	-32.095	140.274
BS379	Y	N	N	N	M	OR	2011	NA	-32.094	140.274
R60607	Y	N	N	N	F	OR	2005	NA	-32.067	140.333
BS398	Y	N	N	N	M	OR	2011	NA	-31.981	140.208
BS400	Y	N	N	N	F	OR	2011	NA	-31.981	140.210
TG02	Y	N	N	N	M	SFR	2015	NA	-	-
TGG1	Y	N	N	N	M	SFR	2015	NA	-	-
TGO1	Y	N	N	N	M	SFR	2015	NA	-	-
TGOY1	Y	N	N	N	M	SFR	2015	NA	-	-
TGY1	Y	N	N	N	M	SFR	2015	NA	-	-
TG_F8	Y	N	N	N	F	SFR	2010	NA	-	-
R31224	Y	N	N	N	NA	SFR	1987	NA	-32.867	139.933
MR595	Y	N	N	N	M	SFR	2012	NA	-32.843	138.053
MR588	Y	N	N	N	M	SFR	2012	NA	-32.842	138.057
MR590	Y	N	N	N	M	SFR	2012	NA	-32.842	138.057
MR591	Y	N	N	N	M	SFR	2012	NA	-32.842	138.057
AG63	Y	N	N	N	F	SFR	2010	NA	-32.745	138.073

R41137	Y	N	N	N	NA	SFR	1992	NA	-32.592	139.861
DP88	Y	N	N	N	M	SFR	2010	NA	-32.416	137.993
DS141	Y	N	N	N	F	SFR	2010	NA	-32.310	137.980
WG136	Y	N	N	N	F	SFR	2010	NA	-32.188	138.011
WG143	Y	N	N	N	M	SFR	2010	NA	-32.188	138.010
WG166	Y	N	N	N	M	SFR	2010	NA	-32.188	138.009
WG80	Y	N	N	N	M	SFR	2010	NA	-32.188	138.009
WG135	Y	N	N	N	F	SFR	2010	NA	-32.188	138.011
WG146	Y	N	N	N	M	SFR	2010	NA	-32.187	138.008
WG145	Y	N	N	N	M	SFR	2010	NA	-32.186	138.008
YC58	Y	N	N	N	NA	SFR	2010	NA	-31.958	138.377
YC59	Y	N	N	N	M	SFR	2010	NA	-31.958	138.377
YC60	Y	N	N	N	M	SFR	2010	NA	-31.956	138.372
YC127	Y	N	N	N	M	SFR	2010	NA	-31.956	138.372
YC93	Y	N	N	N	M	SFR	2010	NA	-31.956	138.372
YC110	Y	N	N	N	M	SFR	2010	NA	-31.954	138.375
YC104	Y	N	N	N	M	SFR	2010	NA	-31.953	138.374
YC105	Y	N	N	N	F	SFR	2010	NA	-31.953	138.374
R57145	Y	N	N	N	M	SFR	-	NA	-31.938	138.105
R60917	Y	N	N	N	NA	SFR	2005	NA	-31.625	138.594
W_M31	Y	N	N	N	M	SFR	2010	NA	-31.546	138.603
W_M23	Y	N	N	N	M	SFR	2010	NA	-31.538	138.599
W_M22	Y	N	N	N	M	SFR	2010	NA	-31.538	138.599
BG51	Y	N	N	N	M	SFR	2010	NA	-31.418	138.558
R52282	Y	N	N	N	NA	SFR	1999	NA	-31.395	138.811
A535	Y	N	N	N	M	SFR	2012	NA	-31.281	138.582
A529	Y	N	N	N	M	SFR	2012	NA	-31.280	138.583
A504	Y	N	N	N	M	SFR	2012	NA	-31.280	138.584
A510	Y	N	N	N	F	SFR	2012	NA	-31.279	138.591
A518	Y	N	N	N	M	SFR	2012	NA	-31.279	138.593
A47	Y	N	N	N	M	SFR	2010	NA	-31.277	138.599

R52684	Y	N	N	N	NA	NFR	1999	NA	-30.768	138.707
R51819	Y	N	N	N	M	NFR	1998	NA	-30.611	138.803
R52317	Y	N	N	N	NA	NFR	1999	NA	-30.598	139.133
R52909	Y	N	N	N	NA	NFR	1999	NA	-30.428	139.145
R31653	Y	N	N	N	NA	NFR	1987	NA	-30.267	139.283
R51794	Y	N	N	N	NA	NFR	1998	NA	-30.198	139.233
R52948	Y	N	N	N	M	NFR	1999	NA	-30.121	139.399

Appendix 3.2. Sampling sites along a north-south linear transect through the *C. decresii* contact zone. Distance is kilometres (km) from southernmost site 1. Variation (mean ± SD) and samples numbers (N) for each trait: hybrid index (H_{Index}; Bayesian q-value), mtDNA haplotype, quantum catch of the ultraviolet wavelength sensitive receptor (QC_{UVS}), PC1 scores for throat coloration (T_{PC1}) and dorsolateral phenotype (D_{PC1}).

Ancestry	Site	Dist.	H_{Index}	N	mtDNA	N	QC_{UVS}	T_{PC1}	N	D_{PC1}	N
South	1	0	0.98 ± 0.06	10	1.0	10	0.65 ± 0.21	0.86 ±	22	1.65 ± 0.83	32
South	2	14	0.95 ± 0.13	11	1.0	1	0.53 ± 0.14	0.76 ±	19	1.89 ± 0.90	21
Admixed	3	28	0.44 ± 0.04	11	0.18	6	0.14 ± 0.11	0.30 ±	15	-0.12 ± 0.51	18
Admixed	4	31	0.40 ± 0.05	31	0.0	10	0.22 ± 0.16	0.48 ±	37	-0.19 ± 0.80	51
Admixed	5	35.5	0.33 ± 0.06	10	0.0	6	0.08 ± 0.06	0.24 ±	11	-0.79 ± 0.62	11
Admixed	6	39.5	0.24 ± 0.04	12	0.0	3	0.19 ± 0.12	0.43 ±	19	-0.22 ± 0.60	19
Admixed	7	45.5	0.16 ± 0.03	9	0.0	1	0.09 ± 0.08	0.23 ±	6	-0.39 ± 0.54	6
Admixed	8	50.5	0.16 ± 0.05	7	0.0	4	0.09 ± 0.03	0.14 ±	2	-0.92 ± 0.56	2
North	9	58	0.09 ± 0.01	16	0.0	5	0.18 ± 0.14	0.35 ±	24	-0.59 ± 0.55	24
North	10	71	0.05 ± 0.01	2	0.0	1	0.10 ± 0.04	0.46 ±	3	-0.89 ± 0.58	3
North	11	81	0.04 ± 0.02	9	0.0	4	0.18 ± 0.14	0.42 ±	10	-0.89 ± 0.56	10
North	12	94	0.02 ± 0.02	8	0.0	9	0.28 ± 0.03	0.53 ±	4	-1.18 ± 0.43	4
North	13	140	0.004 ± 0.02	6	0.0	8	0.20 ± 0.12	0.35 ±	13	-0.70 ± 0.57	13
North	14	160	0.004 ±	7	0.0	6	0.20 ± 0.14	0.50 ±	19	-1.28 ± 0.36	19
			Total	149	-	74	-	-	204	-	233

Appendix 3.3. STRUCTURE results summarised in STRUCTUREHARVESTER using the Evanno method for the full dataset of 1333 loci. The selected value of K (number of populations) is denoted with an asterisk.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-96935.67	0.590361	—	—	—
2*	10	-85534.28	4.320	11401.39	8426.593	1950.583
3	10	-82559.483	9.957	2974.797	2381.325	164.040
4	10	-81973.575	837.453	589.69	631.141	0.754
5	10	-80752.744	369.785	1220.831	—	—

Appendix 3.4. Pairwise F_{ST} values between populations [Northern Flinders Ranges (NFR), Southern Flinders Ranges (SFR), Olary Ranges (OR), Contact Zone (CZ), Mainland South (MS), and Kangaroo Island (KI)]. All values are significant at $P < 0.001$.

	NFR	SFR	OR	CZ	MS	KI
NFR	-	-	-	-	-	-
SFR	0.173	-	-	-	-	-
OR	0.211	0.027	-	-	-	-
CZ	0.216	0.089	0.058	-	-	-
MS	0.468	0.374	0.355	0.203	-	-
KI	0.571	0.434	0.293	0.293	0.213	-

Appendix 3.5. Results of throat and dorsolateral phenotype analyses on wild-caught males and captive-bred offspring (both sexes for throat and only males for dorsolateral). Contributions of variables to components in principal component analyses (PC1, PC2), linear discriminant analyses (LD1, LD2) analyses, and results of a *t*-test (T, P) between northern and southern lineages. Throat variables are the proportions of orange, yellow, and blue colours on the throat, and the quantum catch of the UV receptor (QC_{UVS}). Dorsolateral variables are the proportions of orange and yellow colours on dorsal surface, and the number of distinct breaks in the lateral stripe at the neck.

	Population	Variable	PC1	PC2	LD1	LD2	T	P
Throat	Wild-Caught	Orange	54.56	0.82	0.32	-0.82	-5.77	*<0.001*
		Yellow	9.54	69.77	0.09	-0.06	-1.49	0.137
		Blue	35.9	29.41	2.36	-0.78	23.56	*<0.001*
		QC_{UVS}	-	-	0.62	0.6	9.34	*<0.001*
	Captive-Bred	Orange	-	-	-0.09	-0.5	-4.26	*<0.001*
		Yellow	-	-	-0.54	-0.82	-5.69	*<0.001*
		Blue	-	-	0.59	-0.82	2.27	*0.04*
		QC_{UVS}	-	-	1.15	-0.39	8.82	*<0.001*
Dorsolateral	Wild-Caught	Orange	50.06	0.78	0.65	-0.12	-8.75	*<0.001*
		Yellow	0.01	98.21	0.11	1.13	-3.87	*<0.001*
		LS Breaks	49.94	1.01	1.38	0.04	-30.19	*<0.001*
	Captive-Bred	Orange	-	-	-0.29	-1.1	0.81	0.44
		Yellow	-	-	-0.34	0.57	2.53	0.04
		LS Breaks	-	-	-1.3	0.1	19.45	*<0.001*

Appendix 3.6. Parameter estimates for 95% confidence sets (cumulative Akaike weight $w_i \leq 0.95$) of best-ranked cline models fitted using *HZAR* for the hybrid index based on 1333 loci (H_{index}), mtDNA haplotype frequencies (mtDNA), PC1 for dorsolateral phenotype (D_{PC1}), quantum catch of the UV wavelength sensitive receptor (QC_{UVS}), and PC1 for throat coloration (T_{PC1}). Cline centre (c) is the distance (km) from the southern-most sampling site, width (w) is 1/slope, *pmin* and *pmax* are frequencies at cline ends (for genetic loci), μR and μL are mean trait values at cline ends (for quantitative traits), δ and τ are tail parameters (right, left, or mirror; distance from cline centre to tail and tail slope, respectively). Two log-likelihood unit support limits for centre and width are shown as 'low' and 'high'. Akaike weight (w_i) shown has been recalculated based on only the 95% confidence set to sum to 1.

Trait	Model	c	c_{low}	c_{high}	w	w_{low}	w_{high}	p_{min} / μ_R	p_{max} / μ_L	δ_L	τ_L	δ_R	τ_R	δ_M	τ_M	AIC_c	w_i
H_{index}	*pmin*/*pmax* observed, no tails	29.47	23.96	33.92	38.55	24.60	62.00	0.00	0.98	-	-	-	-	-	-	8.18	0.23
	pmin/*pmax* observed, right tail	27.74	20.09	32.12	15.65	3.45	45.15	0.00	0.98	-	-	0.31	0.40	-	-	8.47	0.20
	pmin/*pmax* fixed, no tails	29.00	23.16	33.66	42.59	29.15	66.34	0.00	1.00	-	-	-	-	-	-	8.82	0.17
	pmin/*pmax* fixed, right tail	27.04	20.25	32.17	20.51	10.02	49.76	0.00	1.00	-	-	0.97	0.48	-	-	8.93	0.16
	pmin/*pmax* fixed, mirror tails	28.78	24.15	32.93	32.07	4.90	57.43	0.00	1.00	-	-	-	-	14.43	0.40	10.73	0.07
	pmin/*pmax* estimated, no tails	28.32	23.60	33.32	32.44	14.77	56.92	0.03	1.00	-	-	-	-	-	-	11.07	0.05
	pmin/*pmax* observed, mirror tails	29.45	24.49	33.40	31.63	2.13	57.06	0.00	0.98	-	-	-	-	15.68	0.42	11.16	0.05

	pmin/*pmax* observed, left tail	29.49	23.95	33.92	38.46	24.61	61.93	0.00	0.98	133.57	0.94	-	-	-	-	12.38	0.03
	pmin/*pmax* observed, both tails	26.46	21.51	32.17	16.58	5.11	45.45	0.00	0.98	35.23	0.44	0.31	0.42	-	-	12.87	0.02
	pmin/*pmax* estimated, right tail	27.16	22.46	31.99	20.02	7.81	46.24	0.01	0.98	-	-	0.22	0.51	-	-	13.07	0.02
	Weighted Average	28.41	22.37	33.00	29.77	15.05	55.84	0.00	0.99	4.52	0.036	0.23	0.10	1.75	0.05	-	-
mtDNA	*pmin*/*pmax* observed, no tails	27.70	14.75	28.17	0.80	0.02	21.88	0.00	1.00	-	-	-	-	-	-	4.17	0.29
	pmin/*pmax* fixed, no tails	27.96	14.76	28.12	0.11	0.07	21.80	0.00	1.00	-	-	-	-	-	-	4.17	0.29
	pmin/*pmax* fixed, right tail	27.60	14.29	28.15	1.03	0.17	21.79	0.00	1.00	-	-	47.72	0.81	-	-	4.17	0.29
	pmin/*pmax* observed, right tail	27.68	13.41	28.17	0.86	0.02	21.89	0.00	1.00	-	-	103.41	0.67	-	-	8.58	0.03
	pmin/*pmax* observed, left tail	27.94	14.74	28.17	0.16	0.01	21.88	0.00	1.00	77.57	0.71	-	-	-	-	8.58	0.03
	pmin/*pmax* fixed, left tail	27.76	14.81	28.14	0.62	0.05	21.86	0.00	1.00	27.18	0.59	-	-	-	-	8.58	0.03
	pmin/*pmax* observed, mirror tails	27.75	14.74	28.17	0.67	0.03	21.87	0.00	1.00	-	-	-	-	47.82	0.28	8.58	0.03
	Weighted Average	27.78	14.59	28.17	0.64	0.08	21.85	0.00	1.0	3.35	0.04	17.20	0.26	1.53	0.01	-	-
D_{PC1}	mean/variance estimated, right tail	22.90	16.44	27.89	6.26	0.79	19.52	-1.11	1.77	-	-	0.76	0.00	-	-	502.71	0.77

	mean/variance estimated, mirror tails	23.43	15.96	26.70	39.49	0.92	60.43	-1.66	2.89	-	-	-	-	9.56	0.07	505.10	0.23
	Weighted Average	23.02	16.33	27.61	13.97	0.82	29.02	-1.24	2.03	-	-	0.59	0.00	2.22	0.02	-	-
QC_{UVS}	mean/variance observed, no tails	15.85	14.3	18.93	9.04	0.88	16.49	0.20	0.65	-	-	-	-	-	-	-199.63	0.71
	mean/variance estimated, no tail	16.98	14.2	25.99	10.44	0.00	21.58	0.18	0.64	-	-	-	-	-	-	-195.84	0.11
	mean/variance observed, left tail	15.94	14.2	27.16	5.69	0.75	16.96	0.20	0.65	0.47	0.36	-	-	-	-	-195.63	0.10
	mean/variance observed, mirror tails	14.99	14.0	19.17	4.89	0.55	18.67	0.20	0.65	-	-	-	-	4.16	0.84	-195.59	0.09
	Weighted Average	15.90	14.25	20.48	8.48	0.74	17.27	0.20	0.65	0.04	0.03	-	-	0.39	0.08	-	-
T_{PC1}	mean/variance estimated, no tails	15.21	15.1	16.11	2.75	2.51	6.58	0.40	0.85	-	-	-	-	-	-	-96.94	0.90
	mean/variance estimated, left tail	16.94	14.7	21.00	7.40	1.78	19.96	0.39	0.86	73.72	0.95	-	-	-	-	-92.58	0.10
	Weighted Average	15.38	15.06	16.61	3.22	2.43	7.94	0.40	0.85	7.52	0.10	-	-	-	-	-	-

Appendix 3.7.

Captive breeding and offspring rearing methods

Adult lizards were housed individually in 55 L × 34 W × 38 D cm opaque plastic enclosures containing a layer of sand and two stacked ceramic tiles for shelter and basking. The room was maintained at temperatures and lighting regimes that mimicked natural seasonal variation. During the breeding season, the ambient temperature was 26°C with a 12:12 light:dark light cycle. A heat lamp was provided in each enclosure to generate a thermal gradient and allow animals to attain their preferred body temperature (36°C; Gibbons 1977; Walker, unpublished data). Lizards were fed live crickets and mealworms *ad libitum* three times per week and misted with water for hydration.

Our breeding design included four possible combinations (northern female × northern male, northern female × southern male, southern female × southern male, southern female × northern male) with up to three 'rounds' of mating to produce multiple clutches in one breeding season. Female enclosures contained a nest box in the form of a clear plastic container 17 × 13 × 15 cm L×W×D with in a 3.5 cm circular entrance in the lower corner, filled with a moist 50:50 sand:peat moss mixture sloped upwards to the top of the box. Eggs were collected following oviposition and incubated in sealed plastic containers filled two-thirds with moist vermiculite (volume ratio 5:1 vermiculite:water) and incubated at 28°C (± 0.12°C) until hatching for a 50:50 sex ratio (Harlow 2000). Hatchling lizards were housed individually in 30 × 20 × 10 cm plastic enclosures with fly screen lids. Each enclosure contained two stacked ceramic tiles to provide shelter, paper substrate, water dish, heat lamp and UV lighting. Lizards were fed crickets *ad libitum* and misted with water daily.

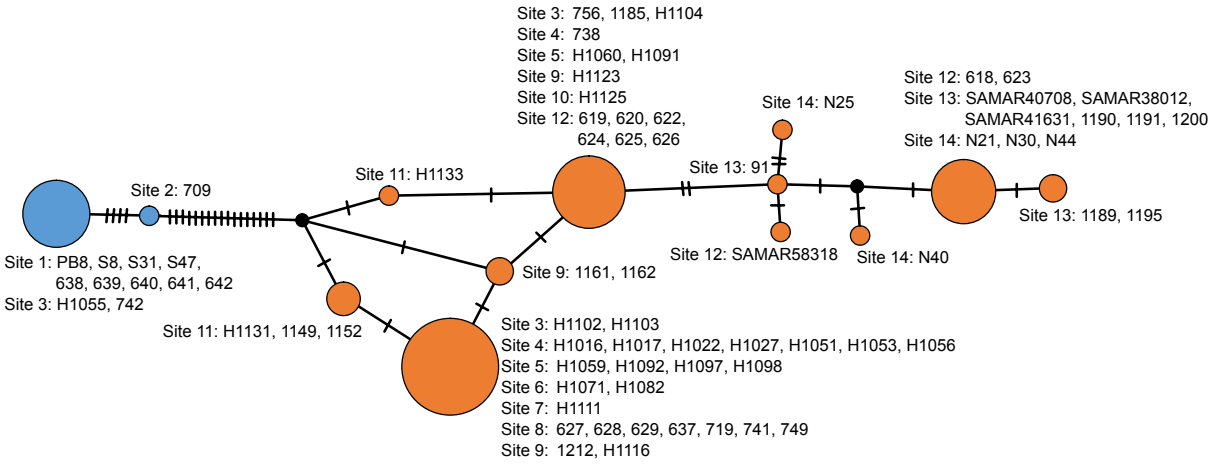
Confirmation of paternity

Maternity was known for all offspring, however paternity was not known due to multiple mates, and the potential for sperm storage and multiple paternity within clutches in the genus *Ctenophorus* (Lebas 2001; Olsson et al. 2007, 2009; Uller et al. 2013; Hacking et al. 2017). We collected blood from all by venipuncture from the sinus angularis in the corner of the mouth. Genomic DNA was extracted from blood using an E.Z.N.A. Tissue DNA Kit (Omega Bio-tek, Norcross, GA, USA) and sent to the Australian Genome Research Facility (Melbourne, Victoria, AUS) for PCR amplification, fragment visualization and size calling. Paternity was assigned with a 95% confidence level using a maximum likelihood approach in CERVUS v 3.0.7 (Marshall et al. 1998; Kalinowski et al. 2007). First, we conducted a simulation of parentage analysis with the parameters of 50,000 offspring, 1% error rate, 5 candidate fathers, 95% of loci typed, and a minimum of 4 loci typed. Following this, we conducted a paternity analysis based on the trio (mother, father, and offspring) LOD score and a strict 95% confidence level (following Rankin et al. 2016). These were manually verified across known breeding pairings.

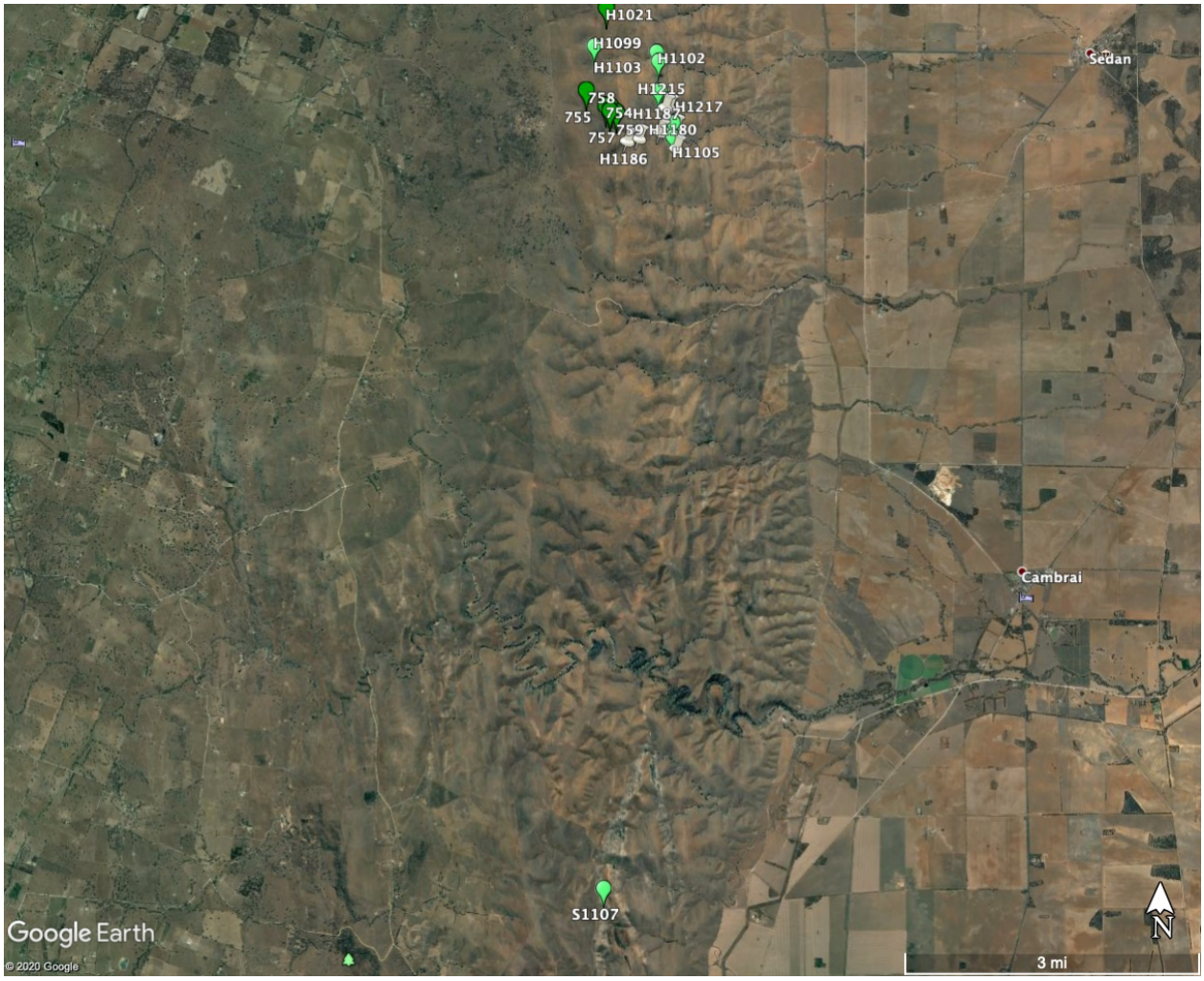
Testosterone-induced throat colour expression

Crystalline testosterone powder (no. T1500, Sigma) was mixed with sesame oil at a concentration of 0.025 g/mL oil and 4.5 uL of this mixture was pipetted onto the dorsal surface daily for six weeks until throat coloration was apparent (Rankin et al. 2016). Ventral and dorsal photos and spectral measurements of throat coloration were taken weekly.

Appendix 3.8. TCS parsimony network of *C. decresii* mtDNA haplotypes constructed from the ND4 region. Circle size corresponds to the frequency of haplotypes and each hash indicates one mutational step. Southern lineage haplotypes are coloured blue, northern lineage haplotypes are orange, and hypothetical missing haplotypes are black. Individuals comprising each circle and their geographic origin (cline site) are indicated by adjacent text.



Appendix 3.9. Satellite imagery of the 12-km density trough between sites 2 and 3, retrieved from Google Earth.



Chapter 4: Maternal reproductive strategy and parental effects on F1 hybrid fitness may influence contact zone dynamics



Caroline M. Dong[1,2], Katrina J. Rankin[1], Claire A. McLean[1,2], & Devi Stuart-Fox[1]

[1]School of BioSciences, The University of Melbourne, Parkville, Victoria, Australia

[2]Sciences Department, Museums Victoria, Carlton Gardens, Victoria, Australia

Photo: Captive-bred juveniles of southern *Ctenophorus decresii*.

4.1 ABSTRACT

The outcome of secondary contact between divergent lineages may be influenced by both the reproductive traits of parental lineages and the fitness of offspring; however, their relative contributions have rarely been evaluated, particularly in longer lived vertebrate species. We performed pure and reciprocal laboratory crosses of the tawny dragon (*Ctenophorus decresii*) to examine how parental reproductive traits and ecologically-relevant offspring fitness traits may explain contact zone dynamics in the wild. The tawny dragon comprises two lineages, northern and southern, which meet in a contact zone of post-F1 hybrids with asymmetric backcrossing and predominantly northern mtDNA haplotypes. We found no evidence for reduced parental investment or offspring fitness for F1 hybrid crosses. However, maternal reproductive strategy and investment differed between lineages, irrespective of the lineage of their mate. Northern females had higher fecundity and produced more and larger clutches with lower embryonic mortality. Parental lineage also influenced sex ratios and offspring traits, with northern ♀ × southern ♂ hybrids exhibiting higher trait values for more fitness measures than remaining offspring. Together, these patterns are consistent with the prevalence of northern lineage mtDNA in the contact zone, and asymmetric backcrossing likely reflects fitness effects that manifest in the F2 generation. Our results highlight how parental lineages can influence multiple offspring traits in different ways, which together may combine to influence offspring fitness and shape contact zone dynamics.

4.2 INTRODUCTION

Hybridisation is an important evolutionary phenomenon that may influence the trajectory or persistence of one or both parental lineages. Although hybridisation may occur across a wide range of spatial and temporal contexts, there are three possible distinct outcomes: a stable equilibrium (i.e. tension zone), the breakdown of barriers due to gene flow and recombination, or the strengthening of barriers driving further divergence (reviewed in Abbott et al. 2013). During secondary contact, the viability and fertility of first generation (F1) hybrids in particular may determine the long-term consequences of hybridisation by precluding, reducing or facilitating gene flow in one or either direction (Wesselingh et al. 2019). Numerous studies have investigated genetic and phenotypic patterns across naturally occurring contact zones but few have concurrently used laboratory studies of parental investment and hybrid fitness to aid in interpreting patterns observed (but see MacGregor et al., 2017; Orr, 1996; Peterson et al., 2005; Rank et al., 2020; Scribner, 1993; While et al., 2015). An interplay of intrinsic and extrinsic factors governs F1 fitness, and laboratory studies (e.g. captive breeding) may enable stronger inferences about the nature of reproductive isolating barriers. For example, the absence of post-F1s across a beetle hybrid zone (*Chrysochus auratus* and *C. cobaltinus*), in conjunction with the reduced fertility of F1 hybrid females in a laboratory setting, suggested that the hybrid zone represented a tension zone (Peterson et al., 2005).

Intrinsic low hybrid fitness is generally attributed to Dobzhansky-Muller incompatibilities (DMIs; Dobzhansky, 1937; Muller, 1942) between biparentally inherited autosomal loci (nuclear-nuclear). Postzygotic barriers are expected to select for the formation of prezygotic barriers to avoid maladaptive hybridisation, thereby promoting speciation (Dobzhansky 1937; reviewed in Butlin 1987; Howard 1993; Noor 1999; Turelli et al. 2001; Servedio and Noor 2003; Coyne and

Orr 2004; Servedio 2004; Ortiz-Barrientos et al. 2009; Abbott et al. 2013; Pfennig 2016). This process of reinforcement following secondary contact is thought to complete speciation initiated in allopatry (Hoskin et al. 2005). Conversely, increased hybrid fitness may result in hybrids outcompeting parental lineages (Rhymer and Simberloff 1996; Huxel 1999; Rosenfield et al. 2004; Taylor et al. 2006), the formation of a stable hybrid zone when hybrids only have higher fitness in the distinct habitat of the contact zone (Nosil et al. 2009) or in tension zones (Barton and Hewitt 1985), or hybrid speciation (reviewed in Mallet 2007). Fitness disparities between reciprocal hybrids may arise from DMIs involving sex chromosomes or cytoplasmic factors (e.g. mitochondria, chloroplasts; (Tiffin et al. 2001; Bolnick and Near 2005; Bolnick et al. 2008; Arntzen et al. 2009; Rank et al. 2020), and may also be shaped by exogenous selection (Campbell and Waser 2001; Kimball et al. 2008; Muir et al. 2015). To distinguish these possibilities, laboratory crosses to assess differences in both parental investment and hybrid fitness are essential.

Here, we investigate differences in parental reproductive investment and the fitness of F1 hybrids produced from captive-breeding between two divergent lineages of *Ctenophorus decresii* (tawny dragon; Duméril and Bibron, 1837) which meet naturally in a zone of secondary contact (Figure 4.1). Two distinct lineages are recognised based on male colour variation and phylogeographic structure: a polymorphic northern lineage with four co-occurring throat colour morphs (orange, yellow, yellow with orange centre, and grey) and a monomorphic southern lineage with blue throats that reflect ultraviolet (UV) wavelengths (Teasdale et al. 2013; McLean et al. 2014b). Secondary contact between populations or lineages that differ in colour morphs is predicted to generate genetic incompatibilities due to the role of colour signals in reproductive isolation (Sætre et al. 1997; Boughman 2001; Jiggins et al. 2001; Mavárez et al. 2006; Seehausen

et al. 2008) and the genetic architecture of colour polymorphism (Sinervo & Svensson, 2002). We recently showed that the polymorphic throat coloration is the target of selection during incipient speciation between the two lineages (Dong et al. 2019a), consistent with the view that colour polymorphism can promote speciation (Corl, Davis, Kuchta, & Sinervo, 2010; Forsman, Ahnesjö, Caesar, & Karlsson, 2008; Gray & McKinnon, 2007; Hugall & Stuart-Fox, 2012; McLean & Stuart-Fox, 2014; West-Eberhard, 1986). The contact zone represents a hybrid swarm and there is evidence of intrinsic incompatibilities, with admixed individuals primarily having the northern lineage mtDNA haplotypes and genomic patterns indicating restricted backcrossing to the southern lineage (McLean et al. 2014b; Dong et al. 2019a). Further, captive behavioural trials indicate incomplete and asymmetric behavioural barriers to mating, with stronger barriers between southern lineage males and northern lineage females than the reciprocal combination (McLean et al. 2020).

In order to assess the nature and extent of reproductive isolating barriers, we conducted a captive-breeding experiment to produce all pairwise combinations of crosses using genetically pure parental populations from either end of the contact zone. We compared parental reproductive life history traits and the viability and fitness of F1 hybrids relative to that of pure lineage offspring. We measured offspring survival, growth rate, maximum bite force, and maximum sprint speed as proxies of competitive ability and fitness, as these traits strongly correlate with ecologically relevant tasks and are known to be the target of selection (reviewed in Irschick et al., 2007; Wainwright, 2007). Based on the structure of the contact zone, we predicted that F1 hybrids would have lower fitness than pure crosses, due to the low prevalence of F1 hybrids and near absence of individuals backcrossed to the south. Furthermore, given the prevalence of the northern mtDNA haplotype in the contact zone, we predicted greater

reproductive investment by northern mothers and/or that hybrids with southern mtDNA haplotypes (i.e. southern mother) may suffer from reduced viability and/or fitness. We discuss the implications of our results for inferring the consequences of secondary contact and trajectories of parental populations.

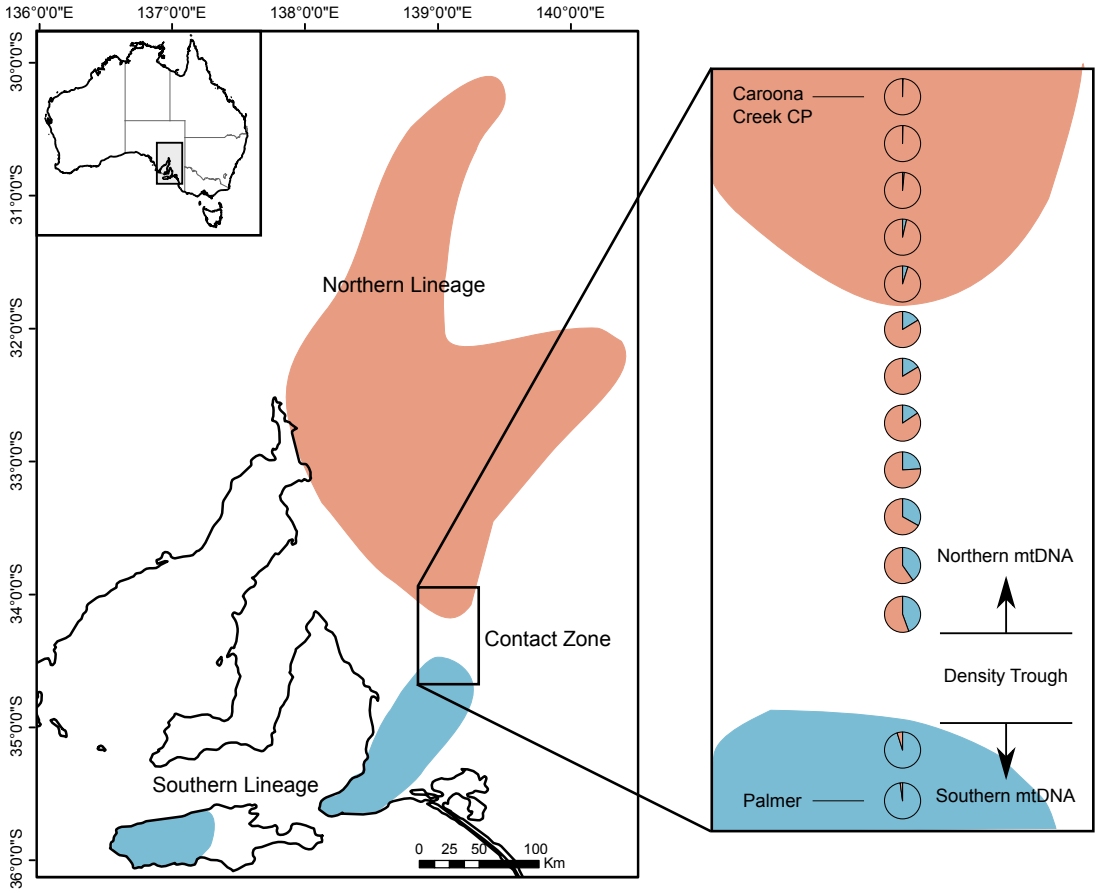


Figure 4.1. The distributions of the northern and southern lineages of *C. decresii* (shaded orange and blue, respectively) and an enlarged map section of the contact zone. Pie charts show mean proportion of genome inherited from each parental lineage (i.e. hybrid index) for 14 sites along a linear transect through the contact zone. An approximately 12-km density trough where multi-year surveys recovered no individuals, and whether sites contain northern or southern mitochondrial (mtDNA) haplotypes, is indicated. Genetically pure sites from which individuals were sourced for captive breeding anchor each end of the contact zone transect: Caroona Creek Conservation Park (CP; northern) and Palmer (southern).

4.3 MATERIALS AND METHODS

4.3.1 Animals

Ctenophorus decresii is a small (snout-vent-length [SVL] ≤ 90 mm) rock-dwelling agamid lizard endemic to South Australia. We captured 36 adult female and 41 adult male *C. decresii* from the wild in 2015 and 2016 (September – November) using a standard lassoing protocol or by hand. This included 18 females and 21 males (northern) from the vicinity of Caroona Creek Conservation Park (longitude: 139.103°, latitude: −33.443°, ca. 130 km north of the contact zone centre) and 17 females and 18 males (southern) from the vicinity of Palmer (longitude: 139.159°, latitude: -34.851°, ca. 30 km south of the contact zone centre) in South Australia (Figure 4.1). These populations represent the nearest pure parental populations to the contact zone. Lizards were housed individually in an indoor facility at The University of Melbourne, Melbourne, Australia, in 55 cm × 34 cm × 38 cm (length [L] × width [W] × depth [D]) opaque plastic enclosures containing a layer of sand and two stacked ceramic tiles for shelter and basking. Lizards were fed live crickets and mealworms *ad libitum* three times per week and misted with water for hydration. The room was maintained at temperature and lighting regimes that reflected natural seasonal variation, with appropriate UV lighting (Outback Max 10.0 UVA/UVB). Heat lamps were provided in each enclosure to generate a thermal gradient and allow animals to attain their preferred body temperature (36°C; Gibbons 1977; Walker, unpublished data).

4.3.2 Captive breeding

Our objective was to produce pure offspring (northern ♀ × northern ♂, southern ♀ × southern ♂) and reciprocal cross hybrids (northern ♀ × southern ♂, southern ♀ × northern ♂). The sexes of each lineage were equally partitioned into two groups (when possible), all individuals of each group were paired with either a same-lineage mate or opposite-lineage mate to produce a clutch of eggs, and this was reversed in the next reproductive cycle for up to three cycles within the breeding season. If a female did not become visibly gravid after two weeks, she was paired with an alternative equivalent-lineage male (typically two males per cycle). One to two days following oviposition, females were housed with a male of the opposite lineage from the previous mate. Males of each lineage followed the same partitioning and pairing procedure such that they encountered alternating lineage females. Breeding trials were conducted in October – December 2017.

Nest boxes were provided in the form of 17 cm × 13 cm × 15 cm (L × W × D) clear plastic enclosed containers with a 3.5 cm circular entrance in a lower corner and damp 50:50 sand:peat moss mixture sloped upwards to the top of the box. For lizards, most females will bury their clutches when provided with a suitable nesting area but inexperienced individuals may lay clutches in the open (Sanger et al. 2008). To account for clutches laid in the open, where they are vulnerable to desiccation, gravid females were checked for evidence of oviposition up to five times daily. Eggs were collected following oviposition (typically within one hour) and individually weighed to the nearest 0.01 g using a digital balance. Eggs were incubated in sealed plastic containers filled two-thirds with moist vermiculite (volume ratio 5:1 vermiculite:water) and incubated at 28°C (± 0.12°C) until hatching. This incubation temperature was chosen for a 50:50 sex ratio (Rankin et al. 2016).

4.3.3 Measuring parental reproductive traits

Indicators of female reproductive investment were: fecundity, clutch size, embryonic mortality, egg mass at oviposition, and offspring mass at hatching. Female fecundity was measured as total reproductive output across the breeding season (i.e. total mass of eggs produced across clutches). Embryonic mortality was scored based on the presence of calcification of the egg shell and/or failure to hatch (Olsson and Shine 1997). Following the breeding season, we recorded male testes mass as this trait may be under sexual selection in lizards (Olsson and Madsen 1998). Males were humanely euthanised, and testes were dissected and weighed to the nearest 0.01 g on a digital balance.

4.3.4 Offspring rearing conditions

Experimental crosses resulted in a total of 53 clutches comprising 259 eggs of which 211 were able to be incubated (i.e. laid in nest boxes or found before they had desiccated). Of these, 176 viable offspring hatched: 69 out of 73 pure northern (94.5%), 14 out of 22 pure southern (63.6%), 61 out of 67 hybrids sired by southern males (91.0%), 32 out of 41 hybrids sired by northern males (78.1%). Offspring were raised for a minimum of six months under laboratory conditions and housed individually in 30 cm × 20 cm × 10 cm (L × W × D) plastic enclosures with fly screen lids. Each enclosure contained two stacked ceramic tiles to provide shelter with paper substrate and a water dish. Ambient temperature was maintained at 24°C, UV lighting was provided, and a heat lamp was positioned at one end of the enclosure for a basking temperature of 31°C. Hatchlings were fed pinhead crickets and fruit flies (*Drosophila*) *ad libitum* twice daily and misted with water. At three months post-hatching, their diet was changed to small crickets *ad libitum* daily, supplemented with mealworms.

4.3.5 Measuring offspring fitness

We recorded offspring SVL (mm) with digital callipers and mass (g) with a digital balance, both to the nearest 0.1, immediately post-hatching and then at weekly intervals for 24 weeks. We used SVL as a measure of growth rate rather than mass because it does not vary with factors such as changes in stomach contents and hydration status. Growth rate from hatching to sexual maturity in reptiles is typically initially linear, before beginning to plateau (Andrews 1982). This was the case for our data, enabling us to use a simple measure of growth rate: $\frac{SVL(24\ weeks)-SVL(hatching)}{elapsed\ weeks}$. Further, overall body condition (i.e. relative body mass) at hatching and 24 weeks of age was calculated by taking the residuals from a linear model of SVL and mass. We recorded deaths in the population for 24 weeks and subsequently quantified the proportion of each group surviving at the conclusion of each week to estimate survival curves.

For both sprint speed and bite force, each lizard was tested at 16 weeks post-hatching during two trials on separate days which consisted of at least two repeated measures per trial. Individuals were not tested for both bite force and sprint speed on the same day. Trials were conducted in the middle of the light cycle (i.e. afternoon) to maximise the likelihood that they had reached their optimal body temperature and reduce variance in temperature. Body temperature was recorded via a thermocouple in the cloaca and the small variation in body temperature did not predict maximum bite force or sprint speed, therefore it was subsequently dropped from analyses (sprint speed: $\chi^2 = 104.34$, *d.f.* = 136, P = 0.98; bite force: $\chi^2 = 23.87$, *d.f.* = 145, P = 1). To quantify morphometric variation relevant to performance, we measured the following morphological characters: head width at the widest point of the head, head length from the tip of the snout to the posterior of the jaw bone, head depth at the deepest point, jaw length from the tip of the snout to the last supralabial scale, hind limb length from the body

wall to the edge of the foot pad, and SVL. All measurements were made using digital callipers to the nearest 0.01 mm. Head dimensions (width, length, depth, jaw) and hind limb length values were regressed against SVL and the residuals were retained for use in subsequent analyses. Principal components analysis (PCA) was performed on the residuals of head dimension measurements to obtain one dimension representing the greatest variance in head shape (principal component 1, PC1). PC1 explained 39.0% of variance and was driven primarily by head length and width (Appendix 4.1).

Sprint trials were conducted in a 115 cm × 10 cm × 20 cm (L × W × D) racetrack on a 30° incline with 80-grit sandpaper as substrate to provide traction and to resemble natural substrate (i.e. rock). A shelter, identical to that provided in their enclosure, was provided at the end of the racetrack. If reluctant to run, lizards were induced to run by gently tapping on the hindquarters using a paintbrush. Trials were filmed using a Canon EOS 600D camera (Canon U.S.A., Inc., Lake Success, NY) at 50 frames per second and subsequently analysed; all trials were conducted and analysed by one observer (C.M.D.). Interval speeds were calculated to 0.01 cm/s over each 10 cm section of the track from trials where the lizard ran continuously for ≥50 cm. Maximum interval speed from between four to six replicates per individual was retained for analysis.

In vivo bite forces were measured using an isometric Kistler Type 9203 force transducer mounted on a vertical holder and connected to a Kistler Type 5995A charge amplifier (Kistler Inc., Winterthur, Switzerland). Thin metal plates (i.e. bite plates) were attached to the force transducer and padded with medical tape on the outer surfaces to prevent damage to the teeth. The mouth of the lizard was opened by gently pulling on the gular region. The open jaws were

placed onto the bite plates at the midway point of jaw length to standardize the point of force exertion; the position and angle of the head was kept as similar as possible across individuals (Lappin and Jones 2014). This provoked voluntary forceful biting, otherwise the side of the mouth was tapped softly to induce biting. At least three recordings were taken per individual and the highest value was retained as the maximum bite force (Herrel et al. 1999).

4.3.6 Confirmation of paternity

Maternity was known for all offspring, however paternity was not known due to multiple mates, and the potential for sperm storage and multiple paternity within clutches in the genus *Ctenophorus* (Lebas 2001; Olsson et al. 2007, 2009; Uller et al. 2013; Hacking et al. 2017). To confirm paternity, we genotyped adults and offspring at five microsatellite loci previously developed for *C. decresii* (Ctde45, Ctde05, Ctde12; McLean et al. 2014) or *C. pictus* and *Amphibolurus muricatus* (CP10 and AM41, respectively; Schwartz et al. 2007). We collected blood for genetic samples by venipuncture from the sinus angularis in the corner of the mouth. Genomic DNA was extracted using an E.Z.N.A. Tissue DNA Kit (Omega Bio-tek, Norcross, GA, USA) and sent to the Australian Genome Research Facility (Melbourne, Victoria, AUS) for PCR amplification, fragment visualization and size calling. Paternity was assigned using CERVUS v3.0.7 (Marshall et al. 1998; Kalinowski et al. 2007) which employs a maximum likelihood approach to assign the most likely candidate sire based on the genotypes of parents and offspring by estimating a likelihood-of-difference (LOD) score. First, we conducted a simulation of parentage analysis with the parameters of 50,000 offspring, 1% error rate, 5 candidate fathers, 95% of loci typed, and a minimum of 4 loci typed. Following this, we conducted a paternity analysis based on the trio (mother, father, and offspring) LOD score and a strict 95% confidence level (following Rankin et al. 2016). These were manually confirmed as

likely fathers based on breeding pairings. To prevent researcher bias, paternity was confirmed following the conclusion of growth and performance data collection.

4.3.7 Statistical analyses

We conducted a linear discriminant analysis (LDA) to assess differentiation between the four classes of offspring lineage (i.e. pure southern, pure northern, hybrids sired by southern males, hybrids sired by northern males). Offspring lineage was used as a dependent variable with fitness traits as independent variables (offspring lineage ~ bite force + sprint speed + growth rate + body condition at 24 weeks + body condition at hatching). Data were centred, scaled, and analysed using the R package *MASS* (v7.3.50; Ripley, 2002).

We used linear mixed models to examine the effects of parental lineage on parental reproductive traits (clutch size, embryonic mortality, seasonality of mating, egg mass at oviposition), offspring fitness traits (offspring mass at hatching, growth rate, bite force, sprint speed, body condition), and offspring morphology (SVL, hind limb length residuals, PC1 for head dimensions). Maternal lineage, paternal lineage, and their interaction were included as fixed terms and unique identifiers for the mother, father, and clutch were included as random-effect terms (response ~ maternal lineage + paternal lineage + maternal lineage*paternal lineage + (1|maternal_ID) + (1|paternal_ID) + (1|clutch_ID)). For body condition, growth rate, bite force, and sprint speed, offspring sex and its interactions with parental lineages were included as fixed terms (response ~ maternal lineage + paternal lineage + sex + maternal lineage*paternal lineage + maternal lineage*sex + paternal lineage*sex + maternal lineage*paternal lineage*sex + (1|maternal_ID) + (1|paternal_ID) + (1|clutch_ID)). For model simplification, we removed non-significant interactions with sex. We examined the residuals of each model for significant departures from

normality and transformed the data if necessary. A Tukey-adjusted least-squares means for pairwise comparisons *post hoc* analysis was performed if an interaction term was statistically significant. One outlier was removed for clutch size and mass (8 eggs from a southern female which required medical intervention to remove them).

To investigate differences in sex ratios, we fit a generalised linear mixed model with a binomial distribution. Offspring sex was the response variable, parental lineages (maternal and paternal) and their interaction were fixed terms, and clutch ID was a random-effect term (sex ~ maternal lineage + paternal lineage + maternal lineage*paternal lineage + (1|clutch_ID)). We examined the relationship between egg mass and hatching mass based on maternal lineage using linear regression. We used the sum of egg masses produced by each female across clutches during the breeding season as a measure of total output (i.e. fecundity) and fit a linear model with fecundity as the response variable and maternal lineage as a fixed term. We extrapolated masses for desiccated clutches from a linear regression of maternal SVL and average egg mass per clutch, for each lineage. To examine offspring survival, we estimated survival curves using the Kaplan-Meier method and compared using a log-rank test (i.e. Mantel-Haenszel test) using the R packages *survival* (Therneau 2015), and *survminer* (Kassambara and Kosinki 2018). Lastly, to investigate differences in testes mass between lineages, we calculated relative testes mass from the residuals of a linear model of SVL and testes mass and then performed a two-sample *t*-test between lineages. All statistical analyses were conducted in R v3.5.1 (R Core Team 2018) using packages *FactoMine*R (Lê et al. 2008), *MASS* (Venables and Ripley 2002), *lme4* (Bates et al. 2015), *lmerTest* (Kuznetsova et al. 2017), and *lsmeans* (Lenth 2016) unless otherwise stated.

4.4 RESULTS

4.4.1 Offspring fitness

Linear discriminant analyses (LDA) showed significant differentiation between the four offspring classes (northern, southern, northern ♀ × southern ♂, southern ♀ × northern ♂; Wilks' $\lambda = 0.545$, $F_{18,331} = 4.4$, $P < 0.0001$; Figure 4.2). LDA showed differentiation by paternal lineage along the first axis (LD1; variance explained = 53.5%; Figure 4.2), which was primarily driven by body condition at 24 weeks of age, bite force, and body condition at hatching (Table 4.1). The second axis (LD2) showed differentiation (variance explained = 43.9%) between maternal lineages driven primarily by growth rate, sprint speed, and body condition at hatching (Table 4.1; Figure 4.2). Correspondingly, univariate analyses revealed significant main effects of maternal lineage on growth rate and sprint speed, and effects of paternal lineage on bite force and body condition at 24 weeks of age in offspring (Table 4.2). Offspring produced by northern females had a higher growth rate in the initial 24 weeks ($P < 0.001$; Table 4.2; Appendix 4.2a) and had higher maximum sprint speeds ($P = 0.007$; Table 4.2; Appendix 4.2b). Offspring sired by southern males had higher maximum bite forces ($P = 0.025$; Table 4.2; Appendix 4.2c) whereas those sired by northern males had higher body condition at 24 weeks of age ($P = 0.004$; Table 4.2; Appendix 4.2d).

There were no significant interactions between parental lineages for offspring fitness traits (growth rate, survival to 24 weeks, bite force, sprint speed, body condition) and morphological characters (SVL, hind limb length residuals, PC1 for head dimensions), indicating that these traits did not differ for pure and hybrid offspring. Additionally, there were no significant effects of parental lineage on body condition at hatching ($P > 0.05$; Table 4.2). There was a significant

effect of sex on growth rate, bite force, body condition at hatching where measures for males were higher than for females (all $P < 0.05$; Table 4.2, Appendix 4.3). Survivorship curves were not significantly different between offspring lineages or by any combination of parental lineage ($P = 0.89$; Figure 4.3e; Appendix 4.4). Parental lineage did not influence any morphological traits ($P > 0.05$; Appendix 4.5).

Table 4.1. Standardised linear discriminant coefficients for the first two canonical variables. Characters strongly correlated with canonical variables (large absolute values) are bolded and italicised.

	LD1	LD2
Bite Force	***-0.531***	-0.107
Sprint Speed	0.385	***0.609***
Growth Rate	0.013	***0.620***
Body Condition at 24 Weeks	***0.831***	0.111
Body Condition at Hatching	***0.550***	***-0.577***
Proportion of variance	0.533	0.439

Table 4.2. Results of a linear model of female fecundity and lineage, and linear mixed-effects models investigating the effects of parental lineage on reproductive traits (embryonic mortality, egg mass at oviposition, offspring mass at hatching) and on offspring fitness traits (growth rate, maximum sprint speed, maximum bite force, body condition at hatching and 24 weeks of age). Statistically significant values are indicated with asterisks (* = P < 0.05, ** = P < 0.01, *** = P < 0.001).

Response	Fixed Term	Sum Sq	Mean Sq	*d.f.*	DenDF	F	P
Fecundity	Maternal	102.10	102.100	1	-	6.45	0.012*
Clutch Size	Maternal	4.01	4.01	1	16.62	4.83	0.042*
	Paternal	0.50	0.50	1	19.95	0.60	0.448
	Maternal × Paternal	0.71	0.71	1	33.29	0.85	0.363
Embryonic Mortality	Maternal	0.32	0.32	1	22.81	6.11	0.021*
	Paternal	0.06	0.06	1	31.37	1.14	0.295
	Maternal × Paternal	0.03	0.03	1	31.37	0.50	0.486
Egg Mass	Maternal	0.11	0.11	1	28.37	21.72	< 0.0001***
	Paternal	0.00	0.00	1	40.69	0.88	0.355
	Maternal × Paternal	0.00	0.00	1	40.69	0.22	0.642
Hatchling Mass	Maternal	0.06	0.06	1	26.53	21.11	< 0.0001***
	Paternal	0.00	0.00	1	40.44	0.49	0.489
	Maternal × Paternal	0.00	0.00	1	40.44	0.06	0.808
Growth Rate	Maternal	0.13	0.13	1	36.37	5.14	0.029*
	Paternal	0.00	0.00	1	26.77	0.03	0.854
	Maternal × Paternal	0.01	0.01	1	39.16	0.41	0.527
	Sex	0.12	0.12	1	125.19	4.59	0.034*
Sprint Speed	Maternal	8370.10	8370.10	1	30.27	9.45	0.004**
	Paternal	2402.10	2402.10	1	25.08	2.71	0.112
	Maternal × Paternal	408.10	408.10	1	32.62	0.46	0.502
	Sex	1.30	1.30	1	114.35	0.00	0.970
Bite Force	Maternal	0.47	0.47	1	35.89	1.01	0.323
	Paternal	2.43	2.43	1	34.08	5.24	0.028*
	Maternal × Paternal	0.24	0.24	1	38.72	0.51	0.478
	Sex	2.70	2.70	1	128.55	5.82	0.017*
Body Condition at Hatching	Maternal	0.02	0.02	1	16.12	1.96	0.180
	Paternal	0.01	0.01	1	25.74	1.09	0.305
	Maternal × Paternal	0.00	0.00	1	24.94	0.37	0.547
	Sex	0.06	0.06	1	124.30	6.88	0.010**
Body Condition at 24 Weeks	Maternal	0.02	0.02	1	19.49	0.12	0.737
	Paternal	1.30	1.30	1	22.40	9.91	0.005**
	Maternal × Paternal	0.06	0.06	1	57.72	0.46	0.501
	Sex	0.06	0.06	1	125.24	0.43	0.511

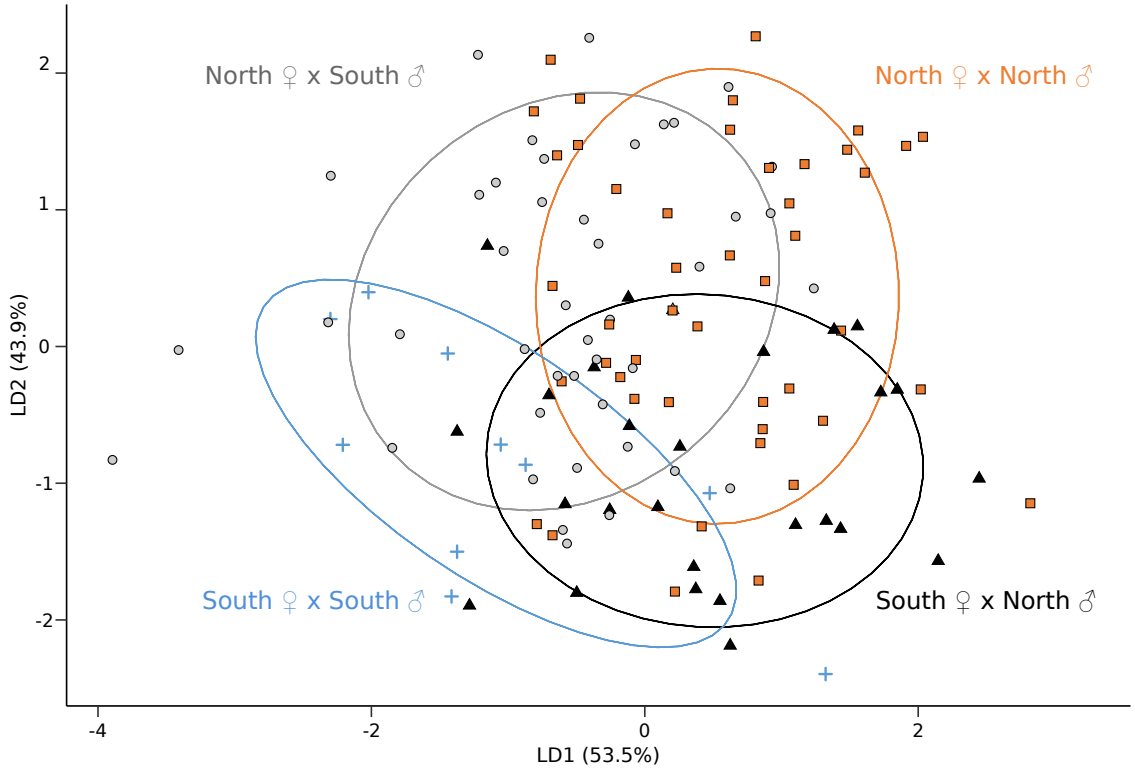


Figure 4.2. Linear discriminant analyses of offspring classes based on five fitness variables: sprint speed, bite force, growth rate, body condition at 24 weeks, body condition at hatching. Linear discriminant 1 (LD1) is positively associated with body condition (at hatching and 24 weeks of age) and negatively associated with bite force; linear discriminant 2 (LD2) is positively associated with growth rate and sprint speed, and negatively associated with body condition at hatching (Table 4.1). 95% confidence ellipses are shown.

4.4.2 Parental reproductive traits

All females were reproductively active: northern females produced 32 clutches comprising 169 eggs and southern females produced 21 clutches comprising 90 eggs. Of these, 5 clutches (29 eggs) and 7 clutches (27 eggs) produced by northern and southern females respectively were found desiccated outside of the nest box and excluded from analyses. A higher proportion of northern females multi-clutched with 66.7% and 11.1% laying second and third clutches

respectively, whereas 23.5% of southern females laid a second clutch and none laid a third clutch.

There were no significant interactions between parental lineages for reproductive fitness traits (fecundity, clutch size, embryonic mortality, egg mass at oviposition, and offspring mass at hatching), indicating that these traits did not differ for pure and hybrid pairings. However, maternal lineage affected all these traits. Northern females had overall higher fecundity over the breeding season ($P = 0.012$; Appendix 4.6a). Northern females also produced larger clutches ($P = 0.042$; Table 4.2; Appendix 4.6b) with lower embryonic mortality ($P = 0.021$; Table 4.2; Appendix 4.6c). Southern females produced heavier eggs ($P < 0.001$; Table 4.1; Appendix 4.6d) and correspondingly heavier hatchlings ($P < 0.001$; Table 4.2; Appendix 4.6e). Additionally, for the relationship between egg and hatchling mass, there was a steeper slope and stronger correlation for southern females (slope ± SE = 1.12 ± 0.09, $r = 0.84$) than for northern females (slope ± SE = 0.60 ± 0.06, $r = 0.72$; Appendix 4.6f).

Overall 76.2% and 52.4% of northern and southern males were reproductively successful, siring 26 and 27 clutches total, respectively. There was no significant difference in testes mass between the lineages (*d.f.* = 24.22, T = 0.22, P = 0.83; Appendix 4.6g). We detected a 2.4% rate of multiple paternity, occurring in one out of 41 clutches genotyped. Specifically, a clutch produced by a northern female was sired by two northern males which were paired with the female consecutively in one reproductive cycle and fathered 40% and 60% of the clutch respectively. This low rate of multiple paternity is comparable to the 4.3% rate of multiple paternity estimated from a wild population of northern *C. decresii* (one out of 23 clutches; Hacking et al. 2017). Additionally, there was a significant effect of paternal lineage on sex ratios of clutches

(P = 0.003; Table 4.3; Appendix 4.6h) where clutches sired by northern males had a sex ratio skewed towards males (1 : 1.64 female : male) whereas clutches sired by southern males had a sex ratio skewed towards females (1 : 0.69 female : male) at an incubation temperature of 28°C.

Table 4.3. Results of a generalised linear model investigating the effects of parental lineages on offspring sex ratio. Statistically significant values are indicated with asterisks (* = P < 0.05, ** = P < 0.01, *** = P < 0.001).

Response	Fixed Term	*d.f.*	χ^2	P
Sex	Maternal	1	0.424	0.515
	Paternal	1	8.637	0.003**
	Maternal x Paternal	1	2.362	0.124

4.5 DISCUSSION

Empirical assessments of reproductive incompatibility and hybrid fitness are essential to interpret genomic patterns observed across naturally occurring contact zones. Previous studies of the contact zone between divergent lineages of *C. decresii* revealed a hybrid swarm with predominantly northern lineage mtDNA haplotypes and genetic incompatibilities when backcrossing to the southern lineage (Dong et al. 2019a). In order to investigate intrinsic reproductive barriers, we conducted captive-breeding experiments between individuals from pure populations nearest to the contact zone and measured offspring fitness traits. We found that northern and southern females differed in maternal reproductive investment and strategy, irrespective of the lineage of their mate. Northern females exhibited higher fecundity over the breeding season and produced larger clutches with lower individual egg masses and more clutches per season; whereas southern females produced fewer, smaller clutches with higher individual egg masses. Maternal and paternal lineages also influenced measures of offspring

fitness; but hybrid offspring did not exhibit higher or lower fitness relative to pure lineage offspring for individual fitness traits. However, the combination of traits inherited from maternal and paternal lineages could result in fitness differences among hybrid classes. Specifically, northern ♀ × southern ♂ hybrids may exhibit higher overall fitness due to the combination of higher growth rates, sprint speeds, and bite forces. The cumulative differences in maternal reproductive investment and strategy and parental effects on fitness could contribute to genetic patterns observed across the contact zone of *C. decresii*.

Differences in maternal reproductive strategy may have broad ecological and evolutionary effects on populations by influencing offspring survival and fitness. Northern females produced a greater number of smaller offspring relative to southern females, and this difference in maternal reproductive strategies reflects a fundamental reproductive trade-off between the number and size of eggs, with total clutch mass functionally constrained (Smith and Fretwell 1974; Brockelman 1975; Kaplan and Cooper 1984). Egg and offspring size are intertwined with a range of factors such as survival and growth rate, and have fitness consequences due to the size-dependent nature of performance traits (Sinervo, 1990b, 1993). Additional differences in embryonic mortality, egg and hatching mass relationship, and sex ratio likely reflect differences in other unmeasured underpinning traits (e.g. yolk volume and content, gestation period, selection on incubation conditions). The differing reproductive strategies of the lineages could be driven by climate-driven selection. Climatic conditions of the higher latitudinal range of the southern lineage (i.e. colder, temperate conditions) may place seasonal time constraints on their breeding season, restricting the time for embryo development and resulting in a higher investment in the first clutch. Comparatively, the range of the northern lineage extends to a lower latitude (i.e. warmer, semi-arid conditions), which may allow for multi-clutching.

Maternal and paternal reproductive traits together may contribute to the prevalence of the northern mtDNA haplotype within the contact zone. Based on our data, we estimate that the number of offspring produced by northern females is at least 2–3 fold higher and with lower rates of embryonic mortality than southern females. Additionally, in the context of hybrid crosses within the contact zone, northern ♀ × southern ♂ crosses will result in a higher proportion of female offspring that will pass on northern mtDNA; however, this may vary with differing incubation conditions in the wild. Similarly, differences in parental life-history traits between fishes (*Gambusia affnis* and *G. holbrooki*) were found to confer a competitive advantage (i.e. body size, growth rates, age at sexual maturity) to progeny with *G. holbrooki* maternal parentage (pure and F1 offspring; Scribner 1993). This competitive advantage correlated to patterns in experimental and natural populations where *G. holbrooki* mtDNA predominated (Scribner, 1993; Scribner & Avise, 1994).

Although parental lineage influenced offspring fitness traits, this was independent of the lineage of the other parent. In other words, contrary to our predictions, hybrids did not differ from pure lineage offspring in individual fitness traits. Nonetheless, there was some evidence of differentiation between reciprocal hybrid classes when considering the full complement of fitness traits. Hybrids sired by southern males had higher growth rates and sprint speeds (*via* maternal influences) and higher bite forces (*via* paternal influence). Pure northern offspring had higher growth rates and sprint speeds but lower bite forces; pure southern offspring had lower growth rates and sprint speeds but higher bite forces; and hybrids sired by northern males had lower growth rates, sprint speeds, and bite forces. Based on combined traits, pure northern offspring may have higher fitness than pure southern offspring (i.e. higher values of both axes in the multivariate analysis of fitness); however the relative fitness of parental and hybrid classes

ultimately depends on how the traits we measured during early ontogeny affect adult fitness in the wild.

The fitness traits we measured are ecologically relevant and indicative of survival and reproductive success. Higher growth rates are likely advantageous given the direct effects on factors such as body size and age at sexual maturity (Andrews 1982; Ballinger 1983). Differences in growth rates, exclusive of exogenous influences, are likely to reflect genetic differences in physiology and/or behaviour (Dawson, 1975; Hutchinson & Maness, 1979; Porter & Travy, 1983; Sinervo, 1990a; Sinervo & Adolph, 1989). Further, performance traits (i.e. bite force and sprint speed) are tightly linked with competitive ability in ecologically relevant tasks such as predator evasion (survivorship), foraging and prey capture, and the ability to acquire and defend territories (social dominance and reproductive success) in reptiles (locomotor performance: Garland, Jr. et al. 1990; Jayne and Bennett 1990; Robson and Miles 2000; Lailvaux et al. 2004; Perry et al. 2004; Husak 2006; Husak et al. 2006; Peterson and Husak 2006; reviewed in Husak and Fox 2008; bite force: Lailvaux et al. 2004; Huyghe et al. 2005; Lappin and Husak 2005; Anderson et al. 2008; Husak et al. 2009). These traits may be especially important determinants of male fitness in species with a polyandrous mating system where there is little evidence of female choice, such as *C. decresii*.

Northern ♀ × southern ♂ F1 hybrids exhibited higher trait values for more fitness measures than remaining offspring including the reciprocal hybrid cross, southern ♀ × northern ♂. This is consistent with the observed prevalence of parental forms and hybrids with northern mtDNA in the contact zone; however, we cannot exclude the possibility of sterility or reduced fertility of F1 hybrids with southern mtDNA and/or the contribution of exogenous selection. Given

these patterns, the composition of the F2 generation may be dominated by northern mtDNA (progeny of hybrids sired by southern males and/or pure northern offspring). This is speculative because our study was limited to F1 hybrids due to the generation time of the species (≥ 2 years until sexual maturity). However, F2 hybrids often suffer strong negative fitness effects (hybrid breakdown) when recessive DMIs are more likely to manifest and/or cytonuclear interactions are further disrupted (Burton et al., 2006; Edmands, 1999; Ellison et al., 2008; Stelkens et al., 2015; Xue et al., 2019). Fitness can be rescued by backcrossing to the maternal lineage to restore parental nuclear and mitochondrial combinations, whereas backcrossing to the paternal lineage perpetuates the mismatch (Ellison & Burton, 2008). This is a conceivable explanation for the observed extensive backcrossing to the northern lineage by post-F1 hybrids which virtually all have northern mtDNA (Dong et al. 2019a).

In summary, our data indicate that differences in maternal reproductive investment and strategy, together with parental effects on offspring fitness and sex ratio differences, may contribute to patterns of genetic variation in the *C. decresii* contact zone, particularly the prevalence of northern lineage mtDNA. Stronger intrinsic genetic incompatibilities between the two lineages may manifest primarily in the F2 generation. Nevertheless, our results highlight how divergence between parental lineages in reproductive traits, as well as effects on offspring fitness, can combine to shape contact zone dynamics.

4.6 CONCLUSIONS

In summary, our data indicate that differences in maternal reproductive investment and strategy, together with parental effects on offspring fitness and sex ratio differences, may contribute to patterns of genetic variation in the *C. decresii* contact zone, particularly the prevalence of northern lineage mtDNA. Stronger intrinsic genetic incompatibilities between the two lineages may manifest primarily in the F2 generation. Nevertheless, our results highlight how divergence between parental lineages in reproductive traits, as well as effects on offspring fitness, can combine to shape contact zone dynamics.

4.7 ACKNOWLEDGEMENTS

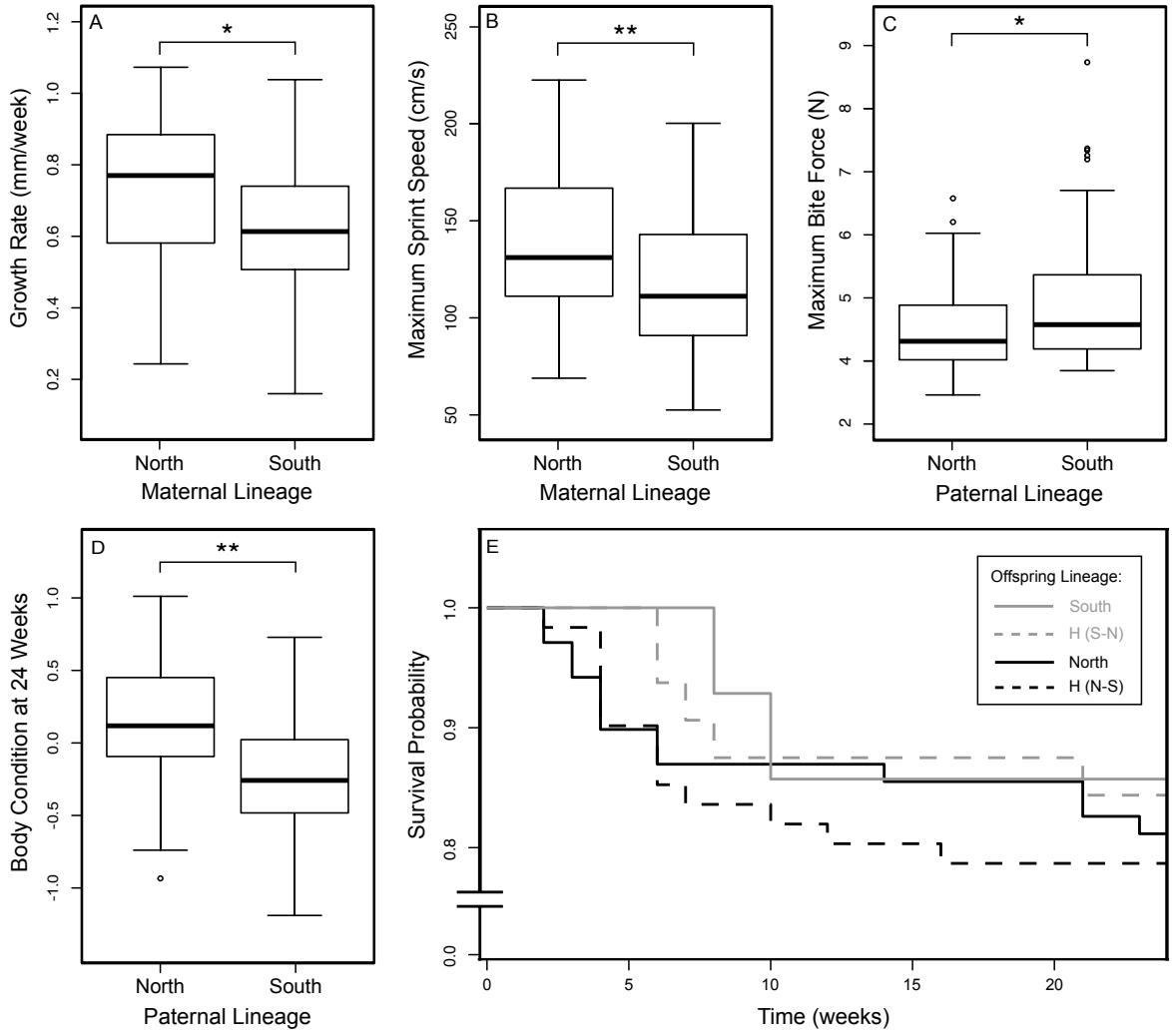
We are grateful to Adam Elliott for fieldwork assistance and lizard husbandry advice; Eliza Thompson, Emma Dragh, Hannah Edwards, Lucy Howell, Miriam Pastor, and Murraya Lane for assistance with captive animal care; Adnan Moussalli for feedback on the manuscript; Po Peng for statistical support; private landowners for access to their property for data collection. This research was conducted with permits from the Department of Environment, Water and Natural Resources, South Australia (permit Q26428-3), the Department of Environment, Land, Water and Planning, Victoria (permit 10007751) and with approval by The University of Melbourne Animals Ethics Committee (approval 1413220.4), and the Wildlife Ethics Committee of South Australia (approval 25/2015).

4.8 APPENDICES

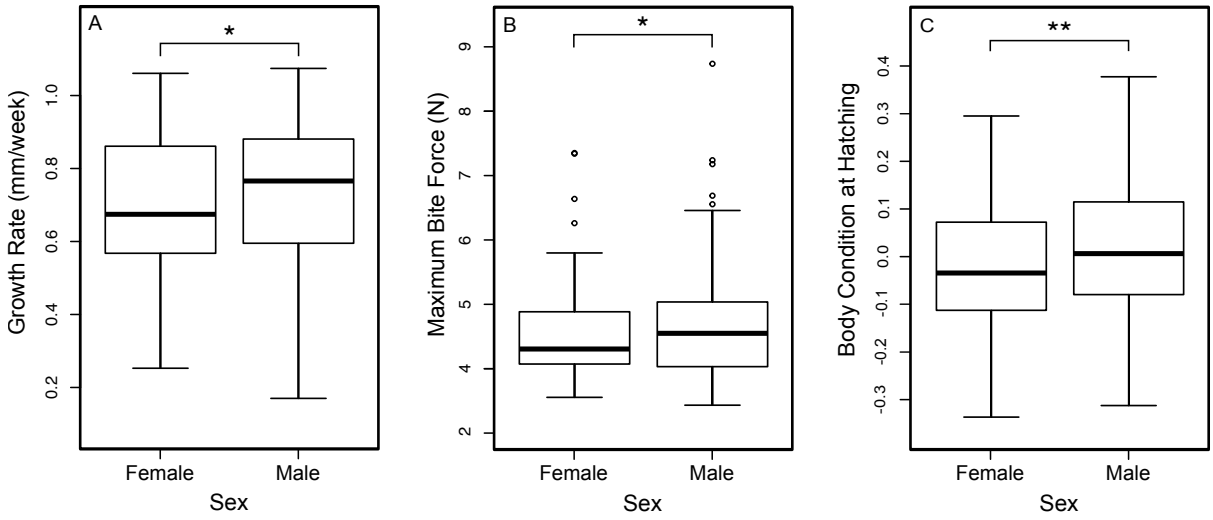
Appendix 4.1. Contributions of head dimension variables in principal components analysis (PCA) and variance explained by each dimension (PC1–4).

Variable	PC1	PC2	PC3	PC4
Head Depth	11.19	40.91	45.01	2.89
Head Length	40.55	4.02	8.95	46.48
Head Width	41.14	2.76	9.31	46.79
Jaw Length	7.12	52.31	36.74	3.84
% Variance Explained	39.0	27.34	20.1	13.5

Appendix 4.2. Lineage differences in measures of F1 hybrid offspring fitness. (a) Growth rate over the initial 24 weeks and (b) maximum sprint speed by maternal lineage; (c) maximum bite force and (d) body condition at 24 weeks of age by paternal lineage; (e) survival curves over the initial 24 weeks estimated using the Kaplan-Meier method by offspring lineage [H (N-S) = northern ♀ × southern ♂ hybrids, H (S-N) = southern ♀ × northern ♂ hybrids]. Statistically significant values are indicated with asterisks (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).



Appendix 4.3. Measures of offspring fitness by sex: (a) growth rate over the initial 24 weeks, (b) maximum bite force, and (c) body condition at hatching. Statistically significant values are indicated with asterisks (* = P < 0.05, ** = P < 0.01, *** = P < 0.001; see Table 4.1).



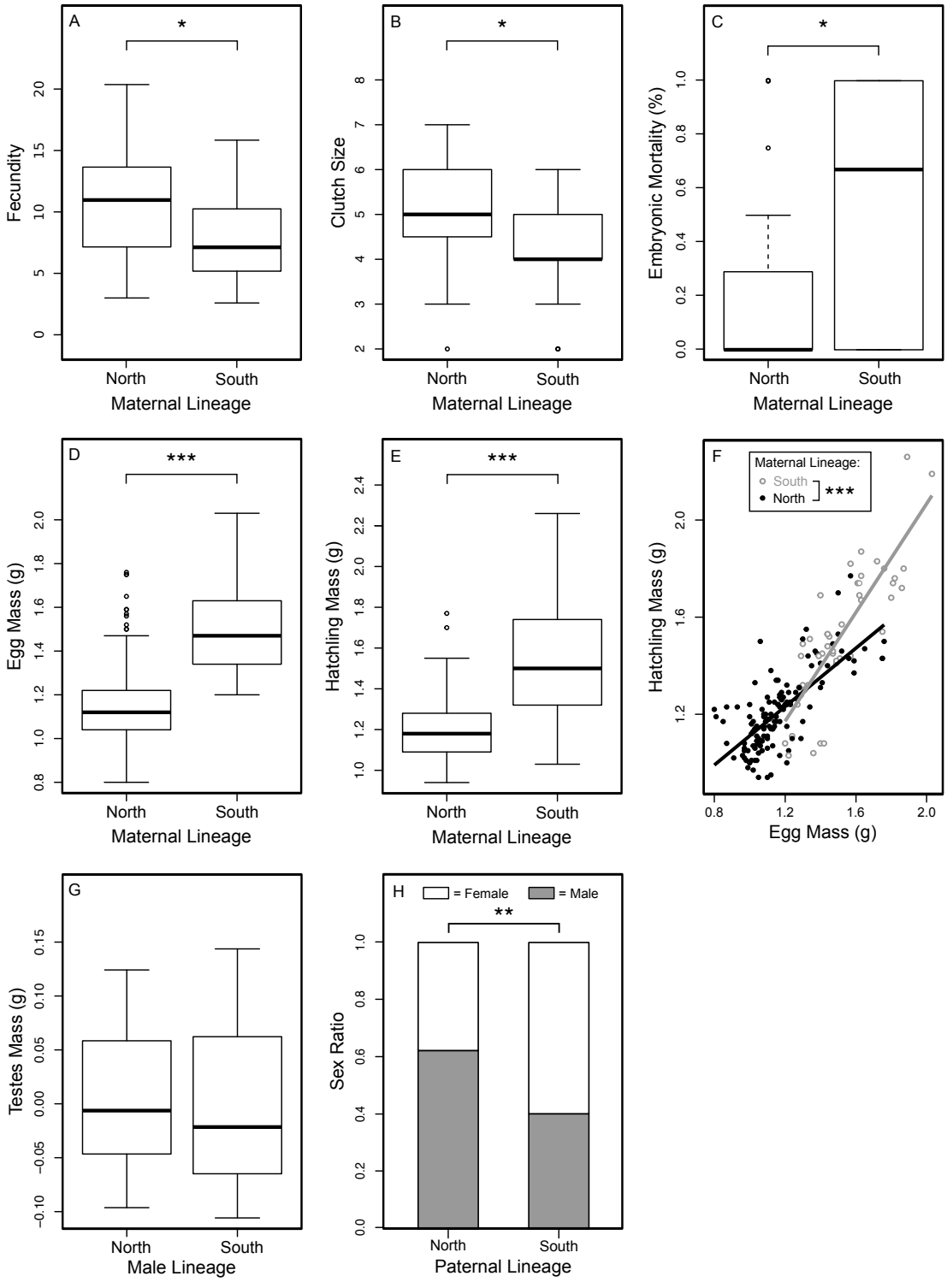
Appendix 4.4. Results of a log-rank test comparing survival over the first 24 weeks between lineages of offspring (X^2 = 0.8, *d.f.* = 3, P = 0.89).

Lineage	N	Observed	Expected	(O-E)^2/E	(O-E)^2/V
Hybrid (N ♀ × S ♂)	61	13	11.21	0.28629	0.44334
Hybrid (S ♀ × N ♂)	32	5	6.24	0.24608	0.31025
North	69	13	12.79	0.00334	0.00557
South	14	2	2.76	0.20875	0.23287

Appendix 4.5. Results of linear mixed-effects models investigating the effects of parental lineage (fixed terms) on offspring morphology: snout-vent-length (SVL), hindlimb length adjusted for SVL, and PC1 from a principal components analysis on head dimensions (head depth, width, length, and jaw length) after adjusting for SVL. Results are not statistically significant.

Response	Fixed Term	Sum Sq	Mean Sq	*d.f.*	DenDF	F	P
SVL	Maternal	0.77	0.77	1	36.701	0.07	0.798
	Paternal	13.01	13.01	1	27.59	1.12	0.298
	Maternal × Paternal	0.34	0.34	1	39.70	0.03	0.864
	Sex	34.17	34.17	1	124.69	2.95	0.088
Hindlimb	Maternal	0.01	0.01	1	23.65	0.01	0.940
Length	Paternal	0.11	0.11	1	40.31	0.06	0.809
	Maternal × Paternal	0.11	0.11	1	39.64	0.06	0.813
	Sex	0.26	0.26	1	133.23	0.14	0.711
Head	Maternal	0.11	0.11	1	27.26	0.12	0.728
Dimensions	Paternal	0.20	0.20	1	34.24	0.22	0.643
	Maternal × Paternal	0.70	0.70	1	32.91	0.75	0.393
	Sex	0.13	0.13	1	119.76	0.14	0.714

Appendix 4.6. Lineage differences in parental reproductive traits: (a) fecundity (i.e. total reproductive output during the breeding season), (b) clutch size, (c) embryonic mortality, (d) egg mass, (e) hatchling mass, and (f) relationship between egg and hatchling masses by maternal lineage with regression line, (g) testes mass adjusted for SVL by male lineage, and (h) sex ratio by paternal lineage. Statistically significant values are indicated with asterisks (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).



Chapter 5: Elevation of divergent colour polymorphic and monomorphic lizard lineages (Squamata: Agamidae) to species level



Caroline M. Dong[1,2], Greg R. Johnston[3,4], Devi Stuart-Fox[1], Adnan Moussalli[2], Katrina J. Rankin[1], & Claire A. McLean[1,2]

[1]School of BioSciences, The University of Melbourne, Parkville, Victoria, Australia

[2]Sciences Department, Museums Victoria, Carlton Gardens, Victoria, Australia

[3]School of Biological Sciences, Flinders University, Adelaide, South Australia, Australia

[4]South Australian Museum, North Terrace, Adelaide, South Australia, Australia

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Photo: *Ctenophorus modestus*, Tothill Belt, South Australia (credit Adam Elliott).

5.1 ABSTRACT

The Australian tawny dragon lizard (*Ctenophorus decresii)*, as currently recognised, comprises two genetically divergent lineages, northern and southern, that differ notably in male coloration. A narrow contact zone exists between the lineages with asymmetric and limited hybridization, indicating incompatibility and highlighting the need for further taxonomic assessment. Here, we evaluate morphological variation in *C. decresii* and elevate the lineages to separate species. The southern lineage retains the name *C. decresii* (Duméril and Bibron 1837), and we formally re-instate *C. modestus* for the northern lineage, which was previously a synonym of *C. decresii* (*Amphibolurus modestus,* Ahl 1926). We re-describe *C. modestus* and *C. decresii* and highlight important considerations for re-evaluation of their conservation statuses. Recognition of *C. modestus* represents another species that may have differentiated in relatively mesic mountainous refugia during Pleistocene glacial-interglacial cycles, and underscores the importance of divergence in male coloration as a driver of speciation in the rock dragon species group.

5.2 INTRODUCTION

Ctenophorus (Fitzinger, 1843) is the largest genus of Australian agamid lizards, comprising a radiation of 33 currently recognised species (Melville and Wilson, 2019; Sadlier et al., 2019). Within the genus there is a monophyletic group of six saxicolous species: *C. decresii* (Duméril and Bibron, 1837), *C. fionni* (Procter, 1923), *C. mirrityana* (McLean et al., 2013), *C. rufescens* (Stirling and Zietz, 1893), *C. tjantjalka* (Johnston, 1992), and *C. vadnappa* (Houston, 1974). All are found in South Australia, with the exception of *C. mirrityana* which is endemic to an adjacent area of western New South Wales. Additionally, all of these species are sexually dimorphic with slightly larger, more brightly coloured and patterned males and cryptically coloured females and juveniles (Houston 1974b; Johnston 1992; McLean et al. 2013). Male throat and dorsolateral coloration are distinguishing characteristics between species and function as important sexual signals (Stuart-Fox and Johnston 2005; Osborne et al. 2012). These traits are emphasised during territorial and courtship displays involving dorsolateral compression of the body and extension of the throat, alongside head-bobbing and hindlimb push-ups (Gibbons 1979; Ramos and Peters 2016).

The tawny dragon (*C. decresii*), comprises two largely allopatric lineages which differ notably in male throat and dorsolateral colour and pattern (Houston 1974b; McLean et al. 2014b). The northern lineage occurs in the Flinders and Olary ranges while the southern lineage occurs in the Mount Lofty ranges, on the Fleurieu Peninsula, and on Kangaroo Island. Divergence between the two lineages is consistent with range contraction to allopatric refugia during glacial-interglacial Pleistocene cycles (Byrne 2008; McLean et al. 2014b) and a secondary contact zone exists near the Barossa Valley, between the Olary and Mount Lofty ranges (McLean et al. 2014b; Dong et al. 2019a). Differing refugium-specific selective pressures may have led to the evolution of divergent phenotypes between lineages (e.g. McLean et al., 2014a). Males of the southern lineage are monomorphic with blue throat

coloration with an ultraviolet (UV) reflectance peak, and some yellow reticulation in the Kangaroo Island population (McLean et al., 2014b; Figure 5.1a –b). The northern lineage is colour polymorphic with four discrete male throat colour morphs: orange, yellow, orange-yellow (yellow with an orange central patch), and grey (Teasdale et al. 2013; Figure 5.1d–g).

McLean et al. (2013 and 2014b) characterised phenotypic and genetic divergence between *C. decresii* (northern and southern), *C. fionni*, *C. mirrityana*, and *C. vadnappa*, and highlighted the need to further investigate the taxonomic statuses of the *C. decresii* lineages. McLean et al. (2014b) found phylogeographic discordance: mitochondrial and nuclear sequence data suggested that populations in the northern Flinders Ranges (northern-most range) were more closely related to the southern lineage than the geographically proximate northern lineage, while this was not supported by microsatellite data. Since then, comprehensive genomic data (1333 single nucleotide polymorphism loci; SNPs) for populations across the range of *C. decresii* has shown that the northern Flinders Ranges populations clearly group with the northern lineage (Figure 5.2). Furthermore, an in-depth study of the contact zone indicates that intrinsic genetic incompatibilities and sexual signal divergence restrict gene flow between the lineages (Dong et al. 2019a). Hybridization is asymmetric and limited; hybrids backcross almost exclusively to the northern lineage and predominantly have northern lineage mtDNA haplotypes and northern throat phenotypes (in terms of coloration and UV reflectance; Dong et al., 2019a). Together, these patterns indicate reproductive incompatibility between the two lineages, which restricts the direction and extent of hybridization.

Here, we formally recognize and re-describe the northern and southern lineages of *C. decresii* as separate species based on notable morphological, phenotypic, and genetic differentiation and evidence of reproductive incompatibilities between the lineages. The type specimens of

C. decresii originate from Kangaroo Island and refer to the southern lineage; *decresii* gives reference to the French name for Kangaroo Island, L'Île de Decrès. The northern lineage is referred to as *Amphibolurus modestus* (Ahl, 1926), previously regarded as a synonym of *C. decresii* by Cogger et al. (1983).

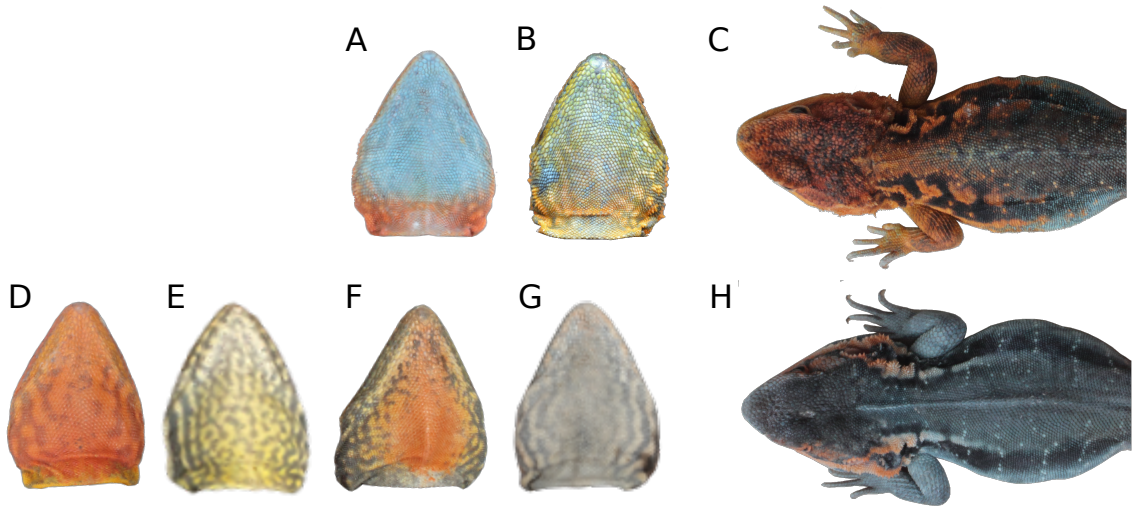


Figure 5.1. a) The blue mainland male throat of *C. decresii* sensu stricto and b) the blue reticulated with yellow throat found on Kangaroo Island. The four male throat morphs of *Ctenophorus modestus*: d) orange, e) yellow, r) orange-yellow (yellow with an orange central patch), and g) grey. Male dorsolateral patterning and coloration differ where c) *C. decresii* sensu stricto has a more "pinched" or broken lateral stripe with a greater extent of bordering orange or yellow coloration, and h) *C. modestus* has a relatively straight edged lateral stripe with cream and orange coloration terminating at the shoulder.

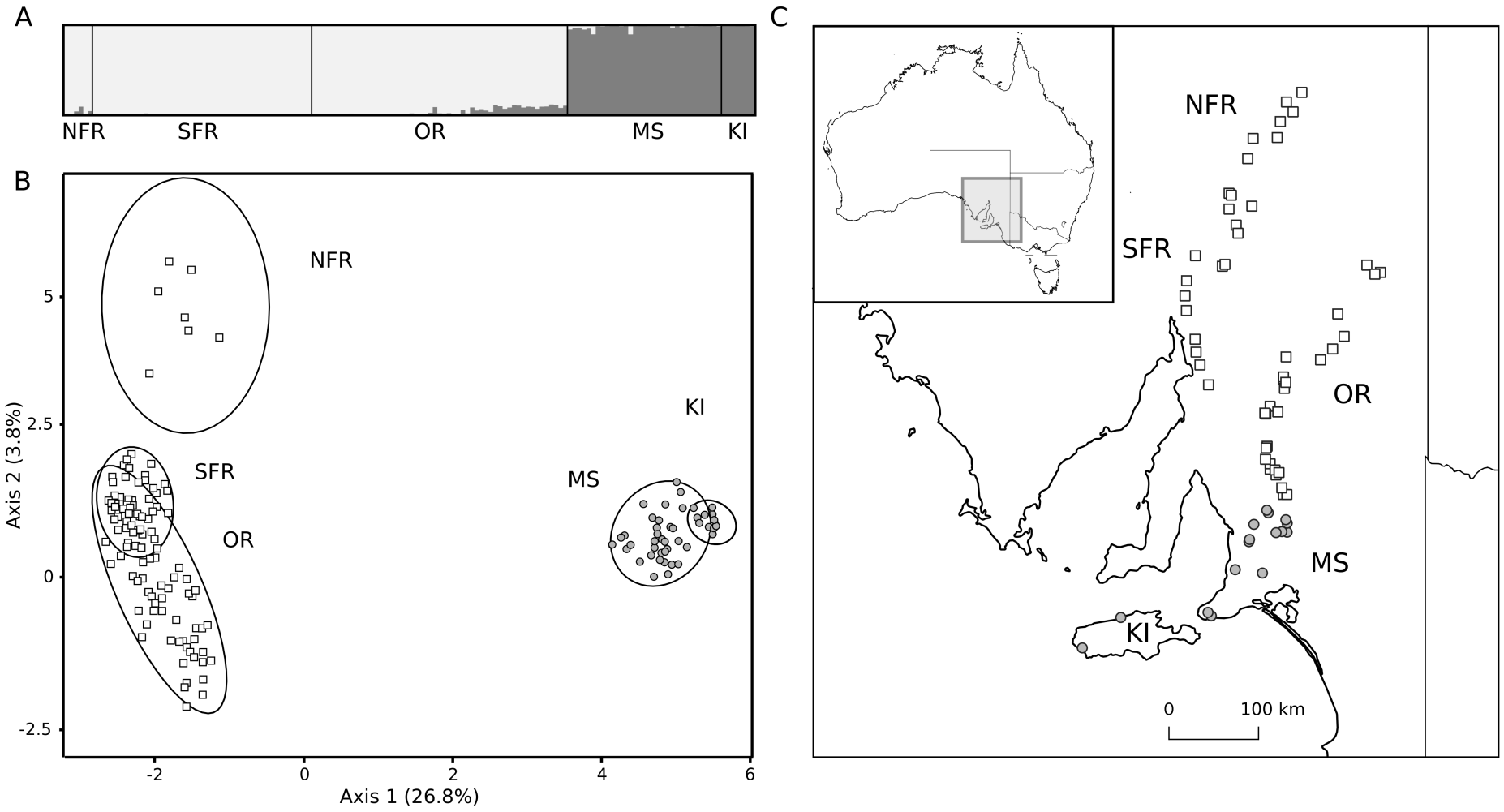

A
NFR
SFR
OR
MS
KI
B
NFR
SFR
OR
KI
MS
Axis 2 (3.8%)
Axis 1 (26.8%)
5
2.5
0
-2.5
-2
0
2
4
6
C
NFR
SFR
OR
MS
KI
0
100 km

Figure 5.2. Genetic divergence between *Ctenophorus modestus* (northern Flinders Ranges [NFR], southern Flinders Ranges [SFR], Olary Ranges [OR]) and *C. decresii* (mainland south [MS] which encompasses the Mount Lofty Ranges and Fleurieu Peninsula, and Kangaroo Island [KI]) and specimens examined for morphological analyses. a) Results of a Bayesian analysis of ancestry in the program STRUCTURE (K = 2). Each vertical bar represents an individual and individuals are ordered by population and latitude (from north to south). The proportion of white and grey represents the proportion of *C. modestus* and *C. decresii* ancestry in each individual, respectively. b) Two-dimensional principal coordinate plot (PCoA) showing pairwise genetic distances between individuals: *Ctenophorus modestus* (□), *C. decresii* (●). 95% confidence ellipses of each population are shown. Both A and B were constructed from a genomic SNP dataset of 1333 SNPs (N = 148). c) Map showing geographic locations of *C. modestus* and *C. decresii* samples shown in A and B.

5.3 MATERIALS AND METHODS

We compared morphometric measurements and meristic characters (Table 5.1) of northern (n = 70; male = 45, female = 25) and southern (n = 40; male = 21, female = 19) *C. decresii*, as well as *C. fionni* (n = 15; male = 10, female = 5), and *C. vadnappa* (n = 15; male = 10, female = 5; Figure 5.3a; Appendix 5.1) as they are the most closely related and morphologically similar species within the rock dragon group (McLean et al. 2013). Data are from McLean et al. (2013) and were collected from adult (SVL ≥ 65 mm for males and ≥ 60 mm for females) preserved specimens held in collections at the Australian Museum, Sydney (AMS), South Australian Museum (SAM), and Museums Victoria (NMV). Full details of data collection and additional univariate data analyses are given in McLean et al. (2013). To assess whether *A. modestus* applied to the northern or southern lineage of *C. decresii*, we obtained photographs, measurements, and meristics of the two syntypes of *A. modestus* (ZMB 30092) held in the Museum für Naturkunde in Berlin, Germany. The syntypes included a subadult male which was re-registered as ZMB54516 in 1995 and a female which retained the original registration number (ZMB30092; Frank Tillack, in lit., 12 December 2019). To confirm that the type specimens of *C. decresii* represent the southern lineage, we obtained photographs, measurements, and meristics of the two syntypes, an adult male (MNHN 6545) and adult female (MNHN 6545A) held in the Muséum National d'Histoire Naturelle in Paris, France. Institutional abbreviations follow Sabaj (2016).

Linear discriminant analyses (LDA) were used to test whether individuals could be reliably assigned to the four *a priori* taxa. Males and females were analysed separately to account for sexual dimorphism, using 23 variables for both (Table 5.1). Morphometric measurements were expressed as a percentage of snout-vent-length (SVL). Tail length (TL) was excluded from both analyses because many individuals had damaged tails (due to predation or intraspecific aggression). Femoral pore count was excluded for females because they could

only be counted reliably for males, and SVL was excluded for males because ZMB54516 is a sub-adult (SVL = 52.9 mm). An additional LDA was conducted on males without the *A. modestus* specimen in order to include SVL as a variable (24 variables used). For all analyses, species was used as a dependent variable (i.e. northern *C. decresii*, southern *C decresii*, *C. fionni*, *C. vadnappa*) with morphological data as independent variables. Data were centred and scaled, and analyses were conducted using the R package *MASS* (v7.3.50; Ripley, 2002) in R v3.5.1 (R Core Team 2018).

Table 5.1. Morphometric measurements and meristic characters used in linear discriminant analysis, including abbreviations and definitions of characters.

Abbreviation	Character
SVL	Snout-vent-length, from tip of rostral scale to cloaca
TL	Tail length, from cloaca to tip of tail
AGL	Axilla-groin length, from base of forelimb to base of
HL	Head length, from tip of rostral scale to posterior of jaw
HW	Head width, at widest point
HD	Head depth, at centre of tympanum
EYE	Eye diameter, from anterior to posterior
SL	Snout length, from tip of rostral scale to anterior of eye
JL	Jaw length, from posterior of eye to posterior of jaw bone
NW	Width between nostrils
HUML	Humerus length
RADL	Radius length
HANL	Hand length, from wrist to tip of fourth finger
FINL	Fourth finger length
FEML	Femur length
TIBL	Tibia length
FOOT	Foot length, from ankle to tip of fourth toe
TOE	Fourth toe length
SUPRA	Number of supralabial scales
INFRA	Number of infralabial scales
ROSNAS	Number of scales between rostral and nasal
SUPRANAS	Number of scales between supralabial and nasal
INTERNAS	Number of internasal scales across the top of the snout
SDL	Number of subdigital lamellae on fourth toe
FP	Number of femoral pores (males only)

5.4 RESULTS

Discrimination between males of the four taxa (northern *C. decresii*, southern *C. decresii*, *C. fionni*, and *C. vadnappa*) was highly significant (males: Wilks' $\lambda = 0.095$, $F_{69,180} = 3.13$, $P < 0.0001$). Linear discriminant analysis (LDA) on male morphological variables indicated that the male type specimens of *A. modestus* and *C. decresii* fell within the 95% confidence ellipses of the northern and southern lineages of *C. decresii*, respectively (Figure 5.3b). Differentiation along the first axis (LD1; variance explained = 52.4%) is driven primarily by number of femoral pores, number of infralabial scales, tibia length, and head length (Table 5.2). The second axis (LD2) showed differentiation (variance explained = 28.5%) between northern *C. decresii*, *C. fionni*, and *C. vadnappa*, driven primarily by tibia length and foot length (Table 5.2; Figure 5.3b). Similarly, discrimination of the four taxa including SVL as a variable (excluding the type specimen of *A. modestus*) for males was highly significant (Wilks' $\lambda = 0.071$, $F_{72,174} = 3.44$, $P < 0.0001$). Differentiation along LD1 (variance explained = 60.7%) is driven primarily by SVL, number of femoral pores, and hand length (Appendix 5.2). LD2 showed differentiation (variance explained = 23.7%) between northern *C. decresii*, *C. fionni*, and *C. vadnappa*, driven primarily by foot length and tibia length (Appendix 5.2, 5.3). In both analyses of males, northern *C. decresii* and southern *C. decresii* were strongly differentiated along LD1 with 95% confidence ellipses that do not overlap (Figure 5.3b, Appendix 5.3). The 95% confidence ellipse of northern *C. decresii* partially overlaps with that of *C. fionni* but not that of *C. vadnappa*; the ellipses of *C. fionni* and *C. vadnappa* partially overlap with each other; the ellipse of southern *C. decresii* does not overlap with any other taxon (Figure 5.3b, Appendix 5.3).

For females, discrimination between the four taxa was also significant (Wilks' $\lambda = 0.104$, $F_{69,90} = 1.48$, $P = 0.039$). LDA on morphological variables showed differentiation along LD1 (variance explained = 45.7%) driven by tibia length, number of scales between rostral and

nasal, and humerus length (Table 5.2; Figure 5.3c). Differentiation along LD2 (variance explained = 34.4%) was driven by SVL, the number of infralabial scales, and hand length (Table 5.2; Figure 5.3c). LDA indicated that the female type specimen of *A. modestus* fell within the 95% confidence ellipse of northern *C. decresii* (Figure 5.3c). The female type specimen of *C. decresii* falls in the overlap between ellipses of southern *C. decresii* and *C. vadnappa* (Figure 5.3c). However, the collection locality of the specimen (i.e. Kangaroo Island) precludes the possibility of the specimen being *C. vadnappa*. The 95% confidence ellipse of southern *C. decresii* partially overlaps with that of both northern *C. decresii* and *C. vadnappa*; however, the ellipses of northern *C. decresii* and *C. vadnappa* do not overlap with each other. The 95% confidence ellipse of *C. fionni* very marginally overlaps with that of *C. vadnappa* only.

Table 5.2. Standardised linear discriminant coefficients for the first two canonical variables for males and females. Characters strongly correlated with canonical variables (large absolute values) are bolded and italicised.

	Males		Females	
Character	LD1	LD2	LD1	LD2
SVL	NA	NA	0.426	***-1.026***
AGL_SVL	0.278	-0.051	-0.313	-0.015
HL_SVL	-0.532	***0.497***	0.058	-0.704
HW_SVL	0.017	-0.366	-0.229	0.033
HD_SVL	0.185	0.312	0.661	0.619
EYE_SVL	-0.062	0.028	-0.426	-0.001
SL_SVL	-0.143	-0.227	0.367	0.354
JL_SVL	0.461	-0.442	0.294	0.204
NW_SVL	0.339	0.331	0.352	0.129
HUML_SVL	-0.158	0.139	***0.753***	0.276
RADL_SVL	-0.405	0.050	-0.679	-0.254
HANL_SVL	0.412	0.284	0.164	***0.825***
FINL_SVL	-0.457	0.311	0.284	-0.411
FEML_SVL	0.487	-0.304	0.684	-0.285
TIBL_SVL	***0.654***	***-0.736***	***-1.024***	-0.612
FOOT_SVL	-0.155	***-0.595***	-0.705	-0.306
TOE_SVL	-0.211	0.058	0.220	-0.110
SUPRA	0.377	-0.208	0.379	0.867
INFRA	***0.665***	***0.503***	0.484	***-0.981***
ROSNAS	-0.232	0.259	***-0.755***	0.029
SUPRANAS	0.408	-0.241	-0.478	-0.653
INTERNAS	-0.114	0.325	0.106	0.542
SDL	-0.181	-0.082	0.059	0.592
FP	***-0.766***	-0.100	NA	NA
Proportion of Variance	0.524	0.285	0.457	0.343

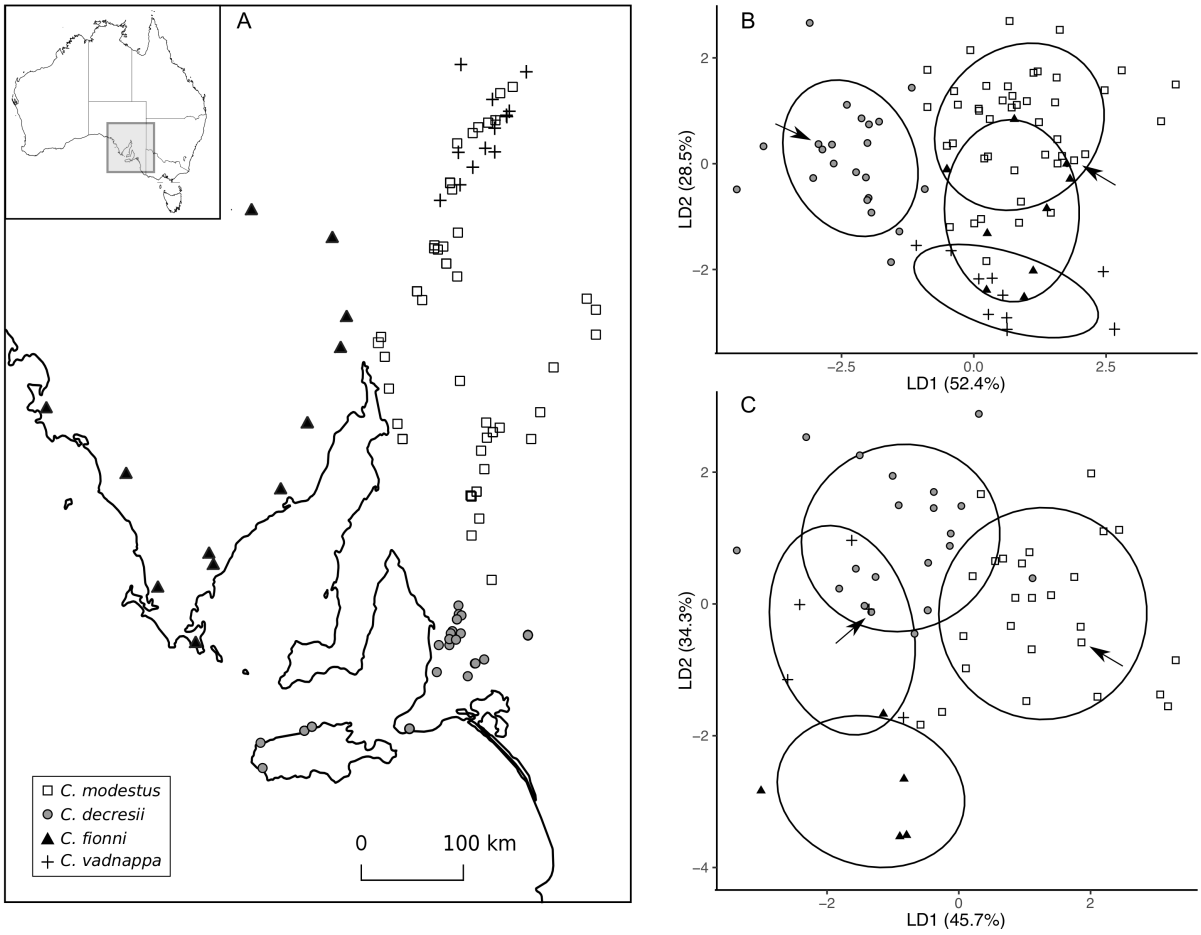


Figure 5.3. a) Map showing localities of examined museum specimens: *Ctenophorus modestus* (□); *C. decresii* sensu stricto (●); *C. fionni* (▲); and *C. vadnappa* (+). Specimens without GPS coordinates are excluded. b–c) Linear discriminant analyses of a) males and b) females based on 23 variables: *Ctenophorus modestus* (□); *C. decresii* (●); *C. fionni* (▲); and *C. vadnappa* (+). 95% confidence ellipses are shown. The lectotypes (males) and paralectotypes (females) of *C. modestus* and *C. decresii* are indicated with arrows.

5.5 TAXONOMY

5.5.1 ***Ctenophorus decresii*** **(Duméril and Bibron, 1837)**

Grammatophora decresii (Duméril and Bibron, 1837, Erpétol. Gen. 4: 472-474)

Amphibolurus decresii 'southern race' (Houston, 1974, Trans. R. Soc. S. Aust. 98: 51)

Ctenophorus decresii 'southern lineage' (McLean et al., 2013, Rec. Aust. Mus. 65: 52)

Tawny rock dragon

Figures 5.1(a–c), 5.4(a–b), 5.5a

Types.–The original description was based on two syntypes, a male and a female, from L'Île de Decrès (Kangaroo Island) and registered as MNHN 6545 (Duméril and Bibron 1837). We designate the male as lectotype (MNHN 6545; Figure 5.5a) and the female as paralectotype (MNHN 6545A; Appendix 5.4) because only the male exhibits the diagnostic coloration.

Diagnosis.–*Ctenophorus decresii* is a member of the *C. decresii* species group (Houston 1974b; Houston and Hutchinson 1998). Within the *C. decresii* species group, *C. decresii* is distinguishable by the following combination of characters: snout scales smooth to longitudinally keeled; vertebral scales flat; presence of a broad dark undulating lateral stripe, often constricted into blotches and interrupted to form two separate blotches posterior to the tympanum; and in males: the throat is blue or blue and yellow with yellow to orange margins.

Description.–A moderately sized lizard reaching a maximum SVL of approximately 88 mm and total length of 230 mm. Head and body depressed. Tail long and tapered to a fine tip. Forelimbs moderately long, almost reaching groin when adpressed. Hindlimbs long and reaching or almost reaching snout when adpressed. Finger lengths: 4 > 3 > 5 > 2 > 1; toe

lengths: 4 > 3 > 5 > 2 > 1. Characteristic of the genus, a distinct row of enlarged keeled scales extending from the nostril, below the eye to above the tympanum (Houston and Hutchinson 1998). Ear membranes exposed and scale-free. Scales on snout are smooth to longitudinally keeled. 12−18 supralabial scales; 11−16 infralabial scales; 4−6 scales between rostral and nasal; 4−6 scales between supralabial and nasal; 10−13 internasal scales; 21–29 subdigital lamellae on the fourth toe (McLean et al. 2013). Dorsal crest is small, no more than a series of keeled scales, becoming smaller posteriorly and not reaching base of tail. Nuchal crest low or absent. Scales on the dorsal surfaces of the limbs and tail are keeled. The gular fold is strongly developed and extends across the shoulders. Forty to forty-six femoral and precloacal pores are arranged in a straight line along the thighs. Pores are present but smaller in females.

Adult male base colour varies from grey-blue to brown, with pale blue flanks (Figure 5.1c, 5.4a). The dorsolateral surface of the head is orange to yellow, extending past the shoulders. The extent of this coloration varies among individuals and depending on location but does not extend further than the mid-point of the axilla-groin length. A broad black lateral stripe begins posterior to the tympanum and terminates at the groin (Figure 5.1c, 5.4a). The lateral stripe forms two separate blotches on the shoulder, one circular and enclosed entirely by orange to yellow coloration, while the other is elongated and wraps partly around the other. The remaining length of the lateral stripe is wavy edged or broken. On the ventral surface, males are cream with orange flushes on the belly, hindlimbs, and the length of the tail during the breeding season. A dark chest patch tapers to a point midbody and sometimes extends along the undersides of the forelimbs. This dark patch may vary in intensity, from black to grey. Male throat coloration in mainland populations is ultraviolet-blue with yellow to orange coloration along the gular fold (Figure 5.1a); in Kangaroo Island populations, the base blue colour is overlain with yellow reticulations (McLean et al., 2014b; Figure 5.1b).

Adult females are cryptically coloured, predominantly grey-brown to orange-brown, densely patterned with darker and lighter speckling (Figure 5.4b) and sometimes a thin, pale vertebral line. Similar to males, a black lateral stripe runs along the flanks, similar in shape to that of males but sometimes less prominently. White to cream on the ventral surface. Varying degrees of grey reticulations on the throat, sometimes with a blue tinge. Orange to yellow flushes on the belly and gular region during the breeding season. Juveniles resemble adult females in coloration and pattern. Colour patterning remains visible in fresh spirit preserved specimens but fades with time.

Measurements (mm) and meristic characters of lectotype (bilaterial meristics as left [L] and right [R]).– SVL, 80; TL, 146.5; AGL, 37; HL, 25; HW, 19.5; HD, 11.5; EYE, 4.7; SL, 9.3; JL, 13.5; NW, 6.5; HUML, 14, RADL, 11.5; HANL, 14; FINL, 10.5; FEML, 17.6; TIBL, 21.3; FOOT, 30.1; TOE, 16.5; SUPRA, 15R/14L; INFRA, 18R/17L; ROSNAS, 5R/5L; SUPRANAS, 5R/5L; INTERNAS, 11; SDL, 30R/NA-L; FP, 24R/21L.

Distribution and natural history.–*Ctenophorus decresii* inhabits rocky areas and outcrops, sheltering in rock crevices. The species occurs in the Mount Lofty ranges, on the Fleurieu Peninsula, and on Kangaroo Island in South Australia, Australia (Figure 5.2c, 5.3a, 5.6). These lizards occupy temperate habitats with varying levels of vegetation. Adults occur almost exclusively on rocks, perching conspicuously on elevated sites to bask and/or perform territorial and courtship displays. They are active in hot weather and if disturbed, will run into a crevice or beneath a rock slab.

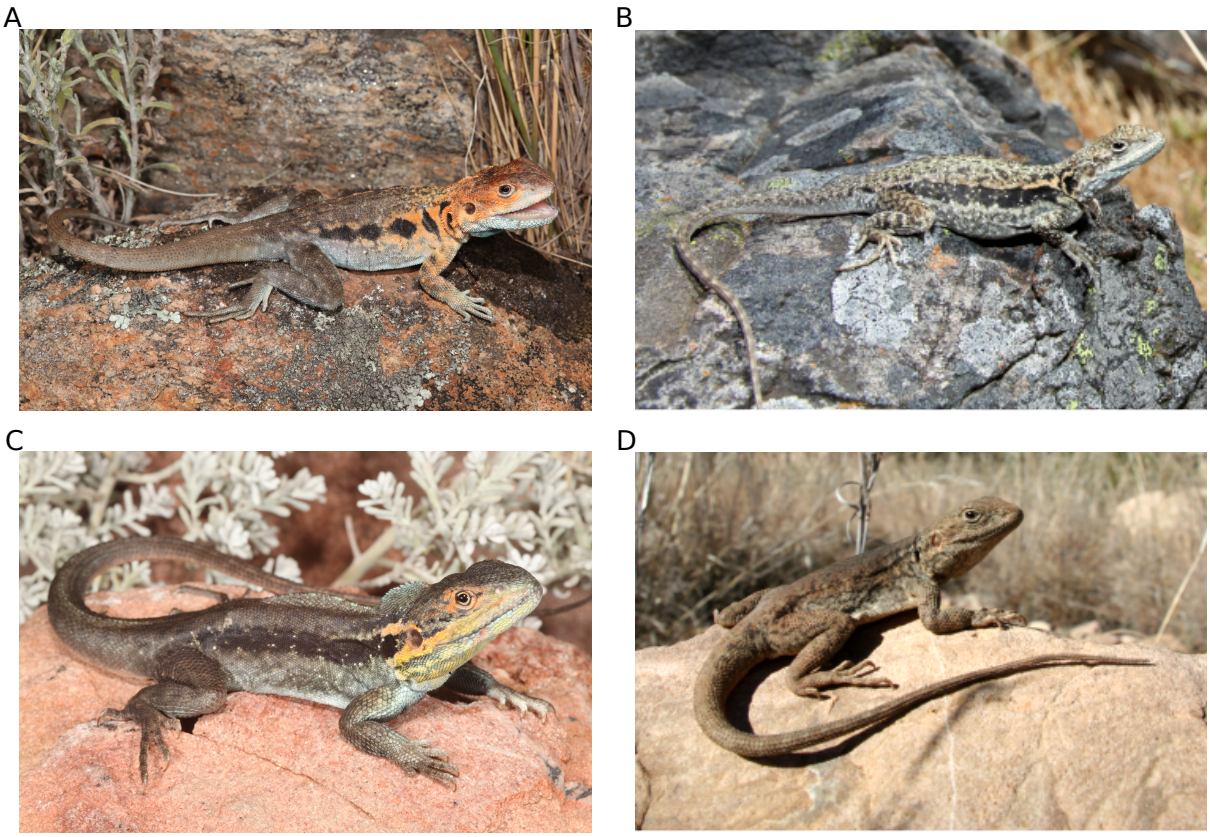


Figure 5.4. Males (a, c) and females (b, d) of *Ctenophorus decresii* (a, b) from Palmer and Mengler's Hill Lookout respectively, and *C. modestus* (C, D) from Telowie Gorge, all from South Australia, Australia (photos Adam Elliott).

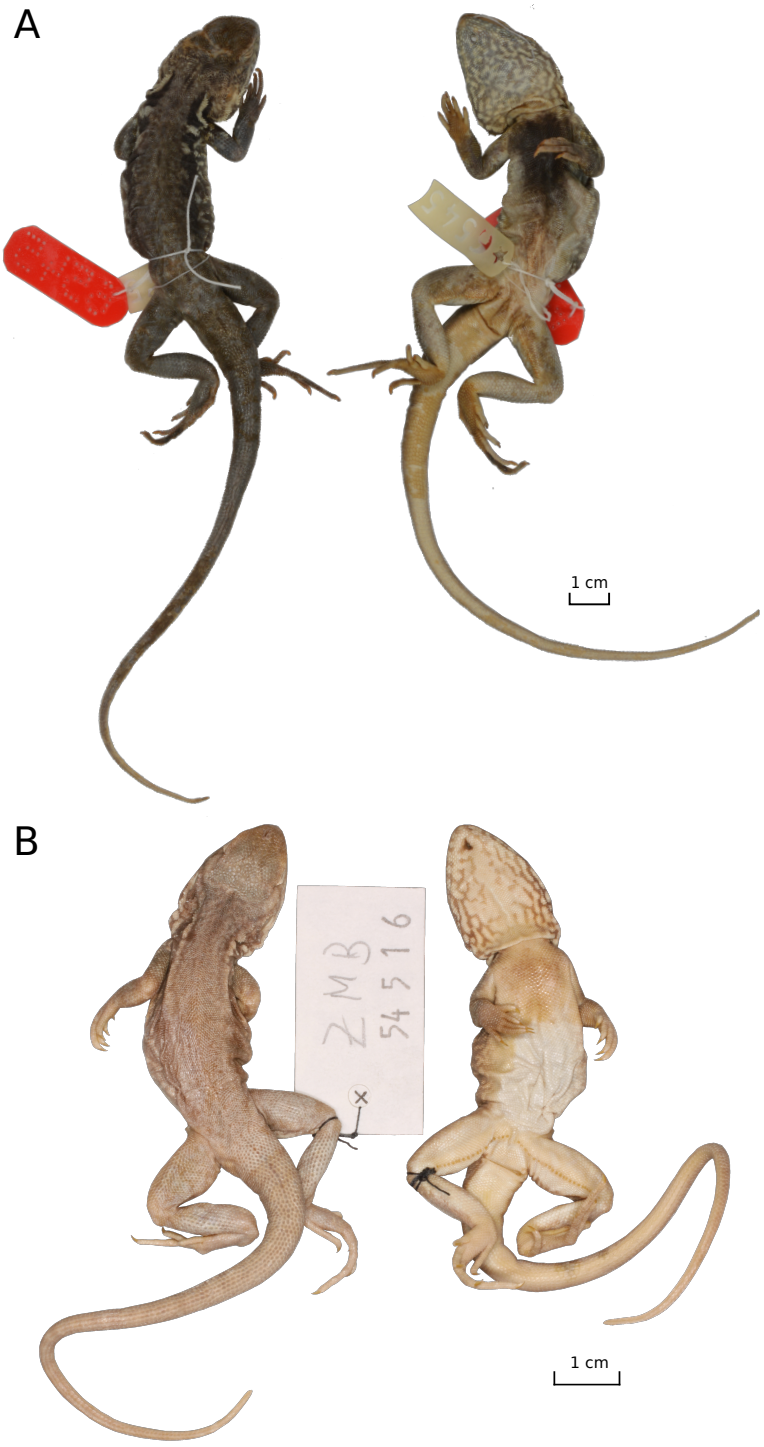


Figure 5. a) Dorsal (left) and ventral (right) photographs of the lectotype of *Ctenophorus decresii* (Duméril and Bibron, 1837; MNHN 6545). This specimen is an adult male collected from Kangaroo Island, South Australia, in January 1803 (photos Nicolas Vidal, Muséum national d'Histoire naturelle Paris). b) Dorsal (left) and ventral (right) photographs of the lectotype of *Ctenophorus modestus* (Ahl, 1926; ZMB 54516). This specimen is a sub-adult male from "Australien" without an exact locality or reference to a collector or donor (photos Frank Tillack, Museum für Naturkunde Berlin).

5.5.2 ***Ctenophorus modestus* (Ahl, 1926)**

Amphibolurus modestus (Ahl, 1926, Zool. Anz. 167: 187-188)

Amphibolurus decresii 'northern race' (Houston, 1974, Trans. R. Soc. S. Aust. 98: 52)

Ctenophorus decresii 'northern lineage' (McLean et al., 2013, Rec. Aust. Mus. 65: 52)

Swift rock dragon

Figures 5.1(d–h), 5.4(c–d), 5.5b

Types.–The original description was based on two syntypes, both from 'Australien' and registered as ZMB30092 (Ahl 1926). We designate the subadult male as lectotype (now registered as ZMB 54516; Figure 5.5b) and the adult female as paralectotype (ZMB 30092; Appendix 5.5) because only the male exhibits the diagnostic coloration. No exact locality is available for these specimens.

Diagnosis.–*Ctenophorus modestus* is a member of the *C. decresii* species group (Houston 1974b; Houston and Hutchinson 1998). Within the *C. decresii* species group, *C. modestus* is distinguishable by the following combination of characters: snout scales smooth to longitudinally keeled; vertebral scales flat; a smooth-edged dark lateral stripe, sometimes interrupted to form a separate blotch posterior to the tympanum; and in males: throat coloration is polymorphic (orange, yellow, orange-yellow, or grey) with or without an overlay of grey reticulations and a small central black patch.

Description.–A moderately sized lizard reaching a maximum SVL of approximately 85 mm and total length of 255 mm. Head and body depressed. Tail long and tapered to a fine tip. Forelimbs moderately long, almost reaching groin when adpressed. Hindlimbs long and reaching or almost reaching snout when adpressed. Finger lengths: 4 > 3 > 5 > 2 > 1; toe lengths: 4 > 3 > 5 > 2 > 1. Characteristic of the genus, a distinct row of enlarged keeled

scales extending from the nostril, below the eye to above the tympanum (Houston and Hutchinson 1998). Ear membranes exposed and scale-free. Scales on snout are smooth to longitudinally keeled. 13−18 supralabial scales; 13−18 infralabial scales; 4−6 scales between rostral and nasal; 4−6 scales between supralabial and nasal; 9−13 internasal scales; 19–27 subdigital lamellae on the fourth toe (McLean et al. 2013). Small clustered pale yellow to orange spines anterior to tympanum. Dorsal crest is small, no more than a series of keeled scales, becoming smaller posteriorly and not reaching base of tail. Nuchal crest low or absent. Scales on the dorsal surfaces of the limbs and tail are keeled. The gular fold is strongly developed and extends across the shoulders. Thirty-four to forty-five femoral and precloacal pores are arranged in a straight line along the thighs. Pores are present but smaller in females.

Adult male base colour varies from grey-blue to brown, with pale blue flanks (Figure 5.1h, 5.4c). The dorsolateral surface of the head is brown, with orange around the eyes, nostrils and along the upper jaw, and beneath the tympanum terminating at the neck. The extent of this coloration varies among individuals and depending on location. A broad black lateral stripe begins posterior to the eye, becoming thicker posterior to the tympanum and terminating at the groin. A thin cream, yellow, or orange stripe begins at the tympanum and terminates just posterior to the shoulder (Figure 5.1h, 5.4c). On the ventral surface, males are cream with orange flushes on the belly, hindlimbs, and the length of the tail during the breeding season. A dark chest patch tapers to a point midbody and sometimes extends along the undersides of the forelimbs. This dark patch may vary in intensity, from black to grey. Male throat coloration varies among individuals with distinct grey, orange, yellow, and orange-yellow morphs (Teasdale et al., 2013; Figure 5.1d–g). Base throat colour sometimes overlain with grey reticulations or with a distinct black mark in the centre of the throat.

Adult females are cryptically coloured, predominantly grey-brown to orange-brown, densely patterned with darker and lighter speckling (Figure 5.4d) and sometimes a thin, pale vertebral line. Similar to males, a black lateral stripe runs along the flanks, but sometimes less prominently than in males. White to cream on the ventral surface, with varying degrees of grey reticulations on the throat. Orange to yellow flushes on the belly and gular region during the breeding season. Juveniles resemble adult females in coloration and pattern. Colour patterning remains visible in fresh spirit preserved specimens but fades with time.

Measurements (mm) and meristic characters of lectotype (bilaterial meristics as left [L] and right [R]).– SVL, 52.9; TL, 95.6; AGL, 24.3; HL, 18.1; HW, 14.0; HD, 7.8; EYE, 3.8; SL, 6.6; JL, 8.3; NW, 5.3; HUML, 7.7, RADL, 8.5; HANL, 11.7; FINL, 5.6; FEML, 14.5; TIBL, 17.4; FOOT, 21.3; TOE, 11.5; SUPRA, 15L/16R; INFRA, 18L/19R; ROSNAS, 4-5L,5R; SUPRANAS, 4L/3-4R; INTERNAS, 12; SDL, 25L/25R; FP, 22L/20R.

Distribution and natural history.–*Ctenophorus modestus* inhabits rocky areas and outcrops, sheltering in rock crevices, similar to other members of the *C. decresii* group (Gibbons 1979). The species occurs throughout the Flinders and Olary Ranges in South Australia, Australia (Figure 2c, 3a, 6). These lizards occupy habitats ranging from well-watered to semi-arid, with sparse vegetation and are active in hot weather (Houston 1974b; Gibbons 1979). Adults occur almost exclusively on rocks but juveniles may be found among surrounding vegetation up to 100 metres away (Houston 1974b; Gibbons and Lillywhite 1981). Adults perch conspicuously on elevated sites to bask and/or perform territorial and courtship displays (Gibbons, 1979). If disturbed, they will run into a crevice or beneath a rock slab. Predators are predominantly birds but likely also include elapid snakes (Gibbons and Lillywhite 1981; Stuart-Fox et al. 2004).

5.5.3 Comparisons between species in the *C. decresii* complex

Similar species.–Members of the *C. decresii* species group differ most markedly in male coloration and geographic distribution. The distribution of *C. modestus* does not overlap with four other species in the *C. decresii* species group (*C. decresii*, *C. mirrityana*, *C. rufescens, C. tjantjalka*). The dorsal colour pattern of *C. modestus* is very similar to that of *C. mirrityana* which occurs in the Barrier Ranges of western New South Wales, but can be distinguished by the absence of a thinner non-continuous orange stripe within the black lateral stripe, absence of a dark median throat stripe in males, and a larger head relative to SVL (McLean et al. 2013). *Ctenophorus tjantjalka* occurs in the low stony hills and eroded creek lines of the Lake Eyre Basin in northern South Australia and has a shorter, deeper head relative to *C. modestus*. Male *C. tjantjalka* are uniform light blue above with salmon flanks and lack the dark lateral stripe of *C. modestus*. *Ctenophorus rufescens* occurs primarily in the granitic ranges of north-western South Australia. Both sexes of *C. rufescens* are rusty to medium brown, lacking both the bright yellow, orange or blue colouring on the head of male *C. modestus* and the dark lateral stripe in juvenile and female *C. modestus*. It is most likely that *C. modestus* may be confused with *C. fionni*, which occurs west of Spencer Gulf and Lake Torrens in South Australia. Male *C. fionni* exhibit extensive geographic variation in coloration (Houston, 1974) and do not differ consistently in any morphological trait from *C. modestus*. Available keys (e.g. Cogger, 2018) differentiate between *C. modestus* and *C. fionni* based on the presence of scattered pale tubercules on the flanks on the former; however, this is primarily diagnostic of *C. decresii* sensu stricto. Additional diagnostic traits are needed to allow *C. fionni* to be distinguished from most other members of the *C. decresii* group independent of a collection locality.

In the northern-most part of its geographic range *C. modestus* occurs in sympatry with *C. vadnappa* (Figure 5.3a). These species can be distinguished by adult male coloration, juvenile

and female coloration, and morphology of the snout and vertebral scales. Adult male *C. vadnappa* have red variegations on the sides of the body that tend to align in vertical bars and no dark lateral stripe, whereas adult male *C. modestus* lack red reticulations and have a dark lateral stripe. Juvenile and adult female *C. vadnappa* also lack the dark lateral stripe, which is present in *C. modestus*. *Ctenophorus vadnappa* have wrinkled snout scales, whereas the snout scales are keeled in *C. modestus*. In *C. vadnappa* the vertebral scales are keeled, whereas they are smooth in *C. modestus*.

There are notable differences in male throat coloration between *C. modestus* and *C. decresii* sensu stricto. Male *C. modestus* have orange, yellow, orange-yellow, or grey throats (Figure 1d–g) whereas male *C. decresii* have blue throats (Figure 1a–b). The black lateral stripe is relatively straight-edged in *C. modestus* compared with the irregular edge in *C. decresii* (Figure 1c, h). *C. modestus* and *C. decresii* also differ significantly in six morphological characters with both sexes of *C. modestus* having higher SVLs (*C. modestus* ♂: 66.3 – 84.6 mm, ♀: 65.1 – 82.9 mm; *C. decresii* ♂: 65.2 – 77.5, mm ♀: 61.9 – 74.13 mm), greater numbers of supralabial (*C. modestus* ♂: 13 – 18, ♀: 13 – 17; *C. decresii* ♂: 12 – 16, ♀: 12 – 18;) and infralabial scales (*C. modestus* ♂: 13 – 18, ♀: 14 – 18; *C. decresii* ♂: 12 – 16, ♀: 11 – 16), and males of *C. modestus* having longer jaws (*C. modestus* ♂: 0.16 – 0.21 mm; *C. decresii* ♂: 0.15 – 0.2 mm; values adjusted for SVL), deeper heads (*C. modestus* ♂: 0.13 – 0.18 mm, *C. decresii* ♂: 0.13 – 0.17 mm; values adjusted for SVL), and fewer femoral pores (*C. modestus* ♂: 34 – 45; *C. decresii* ♂: 40 – 46; McLean et al., 2013).

Conservation status.–*Ctenophorus decresii* (including *C. modestus*) is currently classified as Least Concern by the International Union for Conservation of Nature Red List of Threatened Species (IUCN 2012; Melville et al. 2018). The taxonomic separation of the northern and southern lineages into *C. modestus* and *C. decresii*, respectively, warrants a re-evaluation of

each species' conservation status. Both have distributions that are more restricted than the original distribution of *C. decresii* sensu lato. To measure the extent of occurrence (km^2) for *C. modestus* and *C. decresii* sensu stricto, we drew minimum convex polygons encompassing occurrences based on all specimens in Australian Museums, downloaded from the Atlas of Living Australia. The extent of occurrence for *C. modestus* is 49,102 km^2 (Figure 6), and falls almost entirely within the Flinders Lofty Block (FLB) bioregion as delineated by the Interim Biogeographic Regionalisation for Australia (IBRA version 7; Department of the Environment 2012). The extent of occurrence for *C. decresii* is 6,604 km^2 (mainland: 5,772 km^2; Kangaroo Island: 832 km^2; Figure 6), distributed within both the Flinders Lofty Block and Kanmantoo (KAN) bioregions. The FLB and KAN bioregions consist of 8.9% and 20.3% protected areas (CAPAD 2018b; Table 3). However, the distributions of *C. modestus* and *C. decresii* overlap minimally with protected areas, except in the northern Flinders Ranges and on Kangaroo Island (Figure 6).

The species is locally abundant in suitable habitat in the southern Flinders Ranges and Olary Ranges (Gibbons and Lillywhite 1981); however, suitable rocky habitat is fragmented and most vegetation has been cleared in the southern part of their range. Further, the population of *C. modestus* north of Blinman (31°05'37.1"S 138°40'41"E) in the northern Flinders Ranges is predicted to decline under future climate scenarios (Walker et al. 2014). The IUCN assessment acknowledges a decreasing population trend due to this range contraction (Melville et al. 2018). A better understanding of temporal variation in population density is needed to assess population trends in the northern part of the geographic distribution of both species.

C. decresii may merit conservation management due to its more restricted and fragmented distribution. The total extent of occurrence for the species is less than 20,000 km^2 (i.e. 6,604

km^2). Further, the habitat is fragmented, given that the species occurs only in rocky areas with low to moderate vegetation cover (criteria B1-a). *C decresii* is particularly vulnerable to habitat loss due to human development and bush rock removal (Michael et al. 2010). Although the FLB and KAN bioregions have both experienced grazing pressure from sheep, cattle, and feral goats leading to habitat degradation and clearing, it has occurred more severely in the KAN bioregion (Environment Australia 2000; Bastin 2008). Therefore, *C. decresii* likely has a higher risk conservation status than *C. modestus*. Land clearing has had detrimental impacts on genetic variation in ecologically similar species (Driscoll, 2004; Driscoll & Hardy, 2005; Levy et al., 2010) and vegetation clearance around rock outcrops reduces dispersal between patches of suitable habitat in saxicolous species (Stow et al. 2001; Levy et al. 2010).

Bush rock removal has been implicated in the decline of several threatened reptile species (Michael et al. 2010; Fitzsimons and Michael 2017; Michael and Lindenmayer 2018). Additionally, habitat loss and fragmentation resulting from agriculture creates remnant edge habitats where reptiles experience significantly higher predation risk (Hansen et al. 2019). Furthermore, the degree to which populations of *C. decresii* have been impacted by recent extensive bushfires on Kangaroo Island and in Cudlee Creek on the mainland (ca. December 2019 – January 2020) are still unknown. Although continued monitoring is necessary, the bushfires are likely to negatively impact many subcriteria of B1-b (i–ii: geographic range, iii: area, extent and/or quality of habitat, iv: number of locations or subpopulations, v: number of mature individual).

Table 5.3. Amounts and types of protected areas relevant to the distribution of *Ctenophorus decresii* sensu lato. For the Flinders Lofty Block (FLB) and Kanmantoo (KAN) IBRA bioregions: total area (ha) of the region, total protected area (ha) within the region, percentage of area protected of total area, and percentage of total protected area within each IUCN Protected Area Category: Ia ('Strict Nature Reserve'), Ib ('Wilderness Area'), II ('National Park'), III ('National Monument or Feature'), IV ('Habitat/Species Management Area'), V ('Protected Landscape/Seascape'), VI ('Protected area with sustainable use of natural resources'). Data from CAPAD 2018.

	Total	Protected	%	% Protected Area Per IUCN Category						
Bioregion	Area (ha)	Area (ha)	Protected	Ia	Ib	II	III	IV	V	VI
FLB	6,615,765	591,396	8.9	39.1	0.0	34.6	3.4	0.9	0.0	22.0
KAN	812,414	164,881	20.3	7.0	42.2	23.6	21.9	0.6	0.0	4.4

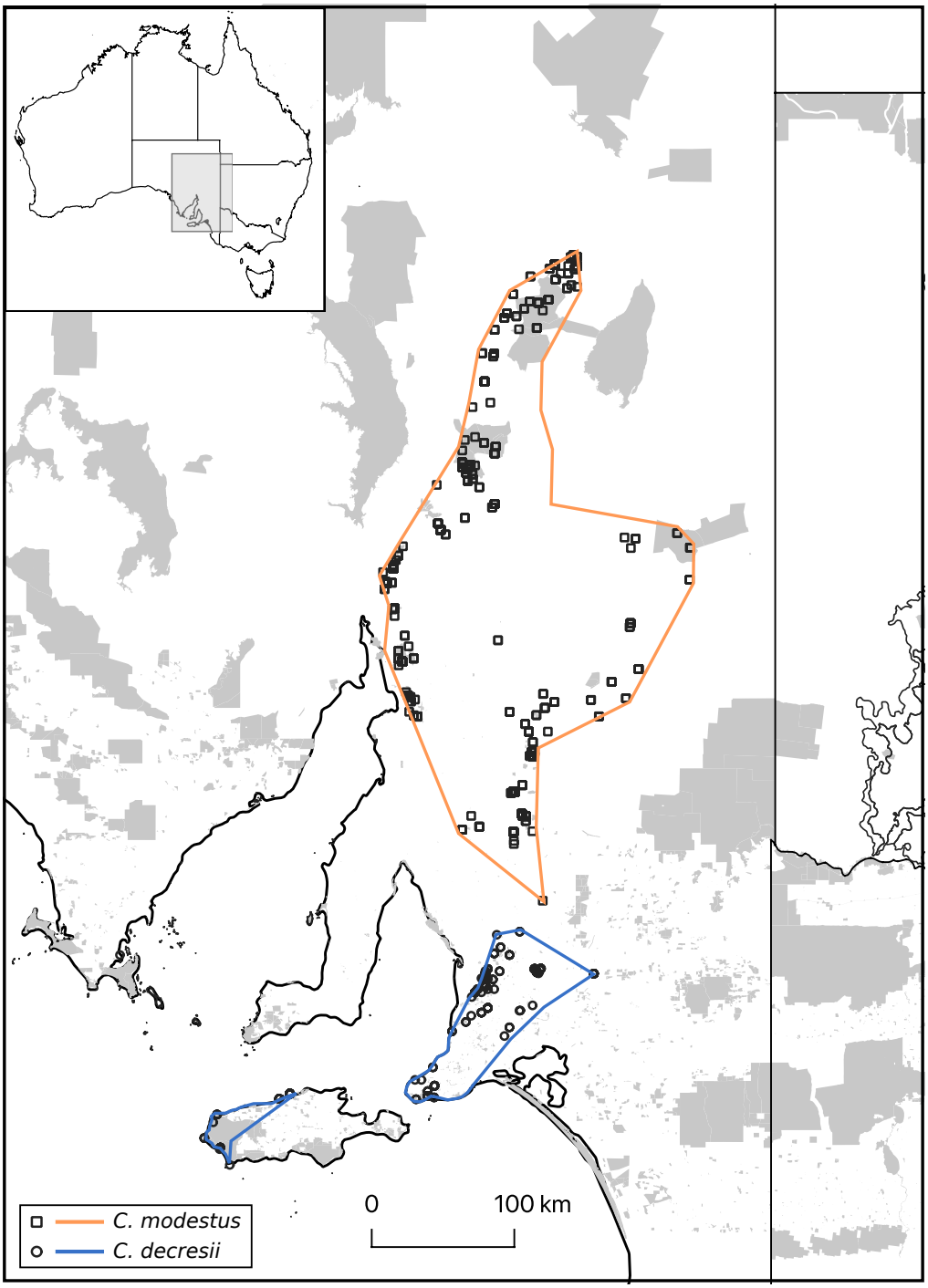


Figure 5.6. Geographic distribution of *Ctenophorus modestus* (□) and *C. decresii* (o) based on specimens in Australian Museums from the Atlas of Living Australia (data: https://doi.org/10.26197/5d91626857226); coordinates which were likely inaccurate (e.g. unsuitable habitat) were removed. Protected areas described in the Collaborative Australian Protected Areas Database (CAPAD, 2018a) are shown as grey areas. The extent of occurrence is shown for *C. modestus* (49,102 km^2; orange lines) and *C. decresii* (6,604 km^2 total [mainland: 5,772 km^2; Kangaroo Island: 832 km^2], blue lines).

Throat polymorphism.–The throat colour polymorphism in *C. modestus* is well-characterised by a large body of literature. Throat coloration is a sexual signal, develops before sexual maturity, is fixed for life and does not vary with body size (Gibbons and Lillywhite 1981; Stuart-Fox 2002), and may function in rival recognition (Osborne et al. 2012). Four discrete male throat morphs (i.e. orange, yellow, orange-yellow, grey) are present in all surveyed populations (Teasdale et al. 2013) with morph frequencies influenced by local environmental conditions (mean annual aridity index and vegetation cover; McLean et al. 2015). The four morphs employ different behavioural strategies, with the grey and orange morphs being the least and most aggressive and bold, respectively, and the yellow morph's behaviour dependent on the competitor's morph (Yewers et al. 2016). These alternative behavioural strategies also correspond to different hormone profiles (Yewers et al. 2017). Throat morphs are associated with MHC supertypes with grey morphs twice as likely to possess supertype four (Hacking et al. 2018).

The genetic and biochemical basis of throat coloration in *C. modestus* has been characterised. Throat morphs are autosomally inherited and controlled by at least two loci. The extent of orange or yellow within morphs behaves as a quantitative (polygenic) trait (Rankin et al. 2016). Orange and yellow skin coloration are produced from a combination of pteridine pigments (self-synthesized) and carotenoid pigments (acquired through the diet), whereas grey skin is produced from melanin pigments (McLean et al. 2017).

Ctenophorus modestus and *C. decresii* share tetrachromatic visual systems with well-developed color vision and no significant differences in spectral sensitivity or opsin expression (Yewers et al. 2015; Dong et al. 2019b). Throat morphs of *C. modestus* vary in conspicuousness, as perceived by agamid lizards (Teasdale et al. 2013). Grey morphs are the least chromatically conspicuous and orange and orange-yellow morphs are the most conspicuous against rock

and lichen backgrounds. The throat colours of *C. decresii* and *C. modestus* are locally adapted to be more conspicuous against native lichen than non-native lichen as perceived by agamid lizards (McLean et al. 2014a).

Biology.–The mating season of *C. modestus* is from September to December. Females produce clutches of between 3 – 7 eggs, commonly laying two clutches in one breeding season (maximum of three clutches; Dong, Rankin, McLean, Stuart-Fox, unpublished). At oviposition, eggs have a mass of 1.16 ± 0.19 grams (mean ± SE) and offspring hatch in 52 – 65 days when incubated at 28°C – 28.5°C (Rankin et al. 2016; Dong et al. unpublished). There are low rates of multiple paternity within clutches: a 4.3% rate (1 in 23 clutches) in a wild population (Hacking et al. 2017), and rates of 3.7% (1 in 27 clutches; Dong et al. unpublished) and 0% (0 in 25 clutches; Rankin et al. 2016) in captive populations. An incubation temperature of 28.5°C yielded a sex ratio of 1 : 0.72 (males : females; n = 58, 3 unsexed; Rankin et al. 2016) and 28°C yielded a sex ratio of 1 : 0.57 (males : females; n = 58, 11 unsexed; Dong et al. unpublished). It is not definitively known whether the species exhibits genetic sex determination (GSD) or temperature-dependent sex determination (TSD). Previous studies on the reproductive biology of *C. decresii* sensu lato demonstrating TSD in the species were conducted on individuals from New South Wales (Harlow 2000; McFadden and Harlow 2007), which have since been re-classified as *C. mirrityana* (McLean et al. 2013). Dragon lizards in the family Agamidae include both GSD and TSD species, with both occurring within the same genera (see references in Ezaz et al. 2009). Further empirical studies are needed to establish the sex-determining mechanisms of both *C. modestus* and *C. decresii*.

Few published studies have focused solely on C. decresii sensu stricto. The species has a similar breeding season of September – December. Females produce one clutch per breeding

season (rarely a second clutch) of between 3 – 6 eggs (Dong et al. unpublished). At oviposition, eggs have a mass of 1.51 ± 0.21 g and will hatch in 57 – 61 days when incubated at 28 ± 0.12°C (mean ± SE; Dong et al. unpublished). There was no evidence for multiple paternity from 14 clutches in a captive population (Dong et al. unpublished), and an incubation temperature of 28°C yielded a sex ratio of 1 : 0.71 (males : females; n = 58, 11 unsexed; Dong et al. unpublished).

5.6 DISCUSSION

Wells and Wellington (1985) have previously resurrected *C. modestus* (Ahl 1926) from synonymy with *C. decresii* and nominated the larger of the two syntypes as a lectotype. However, they provided no rationale or evidence to support this decision. We regard Wells and Wellington's (1985) recognition of *C. modestus* as unavailable for application for the following reasons: (1) they did not provide a "… definition that states in words characters that are purported to differentiate the taxon" as required under Article 13(a)(i–ii) of the third edition of the Code of Zoological Nomenclature applicable at that time; (2) Ahl's (1926) description of *C. modestus* also did not meet this criterion, so Wells and Wellington (1985) also did not provide a "… bibliographic reference to such a published statement… " as required by the same article of the Code. Suggesting that the larger female syntype be regarded as the lectotype suggests Wells and Wellington may not have viewed the syntypes of *C. modestus* because it was well understood at the time that adult male coloration is a key character used to differentiate species in the *C. decresii* group (Houston 1974b; Gibbons and Lillywhite 1981). The importance of male coloration in species diagnosis in this group has become even clearer since then (Johnston 1992; McLean et al. 2013) and is the reason we have nominated the smaller male syntype as the lectotype. While Ahl's (1926) description of *C. modestus* does not provide a clear means of "… differentiating the taxon" required of

publications in 1985, it does conform to the requirements of the Code for species descriptions prior to 1931. Therefore, the name *Amphibolurus modestus* Ahl (1926; now *Ctenophorus modestus*) is available for application. Furthermore, Ahl's male syntype of *C. modestus* is clearly conspecific with the taxon referred to previously as the "northern race" (Houston 1974b) or "northern lineage" (McLean et al. 2013, 2014b) of *C. decresii* sensu lato based on morphology (Figure 3b) and dorsal patterning (Figure 5b). The female syntype is consistent with the "northern race/lineage" of *C. decresii* sensu lato based on morphology, although it is not objectively determinable based on phenotype.

We have resurrected *C. modestus* from synonymy with *C. decresii* on the basis of genetic distinctiveness and diagnostic differences in male coloration, mensural and meristic characters. Historical changes in the distribution of suitable habitat appear to have had an influence on the evolution of the *C. decresii* species group. Extensive rock in the past presumably allowed ancestors of the *C. decresii* group to disperse widely in southern Australia with subsequent isolation leading to the evolution of different species (Houston 1974b). Further, the combination of sexual selection and local adaptation to environmental differences in climate, vegetation and native rock and lichen coloration, have likely contributed to divergence in male coloration between species (McLean et al. 2014a).

5.7 ACKNOWLEDGEMENTS

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5.8 APPENDICES

Appendix 5.1. Morphometric measurements and meristic characters (see Table 5.1) for all museum specimens examined of *Ctenophorus modestus* ("CM"), *C. decresii* ("CD"), *C. fionni* ("CF"), and *C. vadnappa* ("CV").

ID	Species	Sex	SVL	TL	HL	HW	HD	FEML	TIBL	FOOT	TOE	AGL	EYE	SL	JL	NW	HUML	RADL	HANL	FINL	SUPRA	INFRA	ROSNAS	SUPRANAS	INTERNAS	SDL	FP
R62676	CF	M	77.15	NA	28.2	19.27	12.08	19.01	21.82	26.96	14.24	24.01	6.18	9.78	14.8	7.97	13.89	10.44	15.05	8.6	14	15	4	6	11	23	37
R66622	CF	M	72.78	143	25.21	18.77	10.79	18.54	20.38	24.92	14.1	26.2	5.24	9.15	13.69	7.75	12.58	10.31	11.4	6.6	16	16	5	5	11	25	36
R61596	CF	M	83.76	164	28.99	19.8	11.24	21.9	24.91	30.78	15.14	35.15	6.35	9.66	14.15	7.53	12.67	11.5	13.72	7.92	15	14	4	6	11	25	38
R12929D	CF	M	76.18	NA	26.19	18.34	11.37	18.19	22.78	27.03	14.53	30.61	5.72	9.4	12.3	7.44	12.45	11.48	12.78	7.31	15	15	4	4	10	22	38
R12924B	CF	M	70.03	NA	23.66	17.54	11.1	17.34	19.56	25.73	13.55	27.77	5.19	8.93	13.07	7.84	10.5	8.01	12.43	7.73	16	15	5	5	10	26	42
R12924A	CF	M	66.41	150	24.43	17.52	11.1	18.88	21.39	26.89	14.37	23.3	4.86	8.7	12.74	6.94	12.49	9.05	13.86	8.01	15	13	4	5	11	23	40
R12930A	CF	M	71.85	141	24.27	17.5	10.43	19.81	22.08	26.64	14.79	26.71	5.65	8.5	12.72	7.08	12.02	9.89	11.66	7.61	14	15	4	4	11	24	38
R13900B	CF	M	75.05	137	25.99	18.54	11.55	19.4	21.54	26.74	14.88	28.2	5.61	9.75	14.23	7.27	11.9	9.99	10.82	6.82	15	15	4	5	11	23	38
R13054	CF	M	73.27	139	24.23	17.93	10.62	17.59	20.44	25.38	13.64	30.46	5.56	8.77	12.5	6.96	10.97	9.17	10.41	7.02	14	15	5	5	12	23	40
R13323	CF	M	75.97	136	25.9	17.94	11.49	18.92	22.23	28.71	15.2	28.53	5.49	8.82	13.22	7.82	11.61	9.29	13.72	8.74	16	14	5	6	12	24	36
R51778	CV	M	69.08	156	23.5	17.11	10.32	18.27	20.98	27.67	15.35	26.87	5.16	7.94	12.84	7.01	9.09	9.67	13.9	8.1	14	14	4	5	12	23	44

R51805	CV	M	72.87	163	25.72	17.93	10.84	21.42	23.88	27.97	16.07	28.31	5.89	9.19	13.4	7.04	11.59	10.83	12.66	7.53	14	15	4	6	11	23	36
R51905	CV	M	79.91	166	27.7	19.47	11.79	23.13	24.25	30.48	17.18	32.95	6.29	10.36	15.38	8.61	12.12	12.11	12.95	7.8	15	14	4	5	13	27	40
R51916	CV	M	71.62	157	23.97	17.26	10.1	20.65	23.63	27.92	16.73	28.31	5.46	7.73	12.51	6.49	11.88	10.49	13.66	6.95	15	15	5	4	11	23	42
R51970	CV	M	72.73	NA	24.75	18.19	10.78	20.43	22.19	27.63	15.21	26.65	5.53	8.75	13.87	8.08	10.28	9.57	12.98	8.13	16	14	5	5	11	23	38
R52000	CV	M	81.77	182	28.66	20.11	12.5	22.5	24.24	31.55	16.7	32.09	5.51	9.31	16.38	7.41	13.06	10.51	12.52	6.94	14	14	5	5	11	25	40
R52021	CV	M	78.51	165	27.75	20.33	11.98	22.84	24.93	30.78	16.53	32.43	5.39	9.49	15.22	8.36	11.99	11.84	13.48	7.7	14	14	4	5	13	26	43
R52164	CV	M	77.15	165	27.3	19.33	11.86	21.7	23.83	31.12	17.71	29.9	5.65	9.79	14.63	7.84	12.47	12.48	15.14	8.42	15	15	5	5	10	25	40
R52321	CV	M	75.62	168	26.18	18.58	11.14	20.96	24.21	30.06	17.28	34.06	5.53	8.73	13.51	8.32	10.83	11.57	14.14	9.07	14	14	5	5	11	27	44
R64524	CV	M	76.87	166	26.02	19.06	11.13	19.94	22.64	29.89	17.22	31.91	5.69	8.54	14.3	7.46	10.63	11.19	13.8	8.07	16	15	4	6	11	25	45
NMVD3411	CM	M	66.34	129	22.31	15.89	9.74	15.56	17.2	25	13.57	29.02	4.61	7.37	12.16	6.39	10.66	9.06	13.67	7.11	16	16	5	5	11	24	38
R44452	CM	M	78.57	155	27.65	19.36	13.13	19.87	23.47	26.95	15.33	33.45	5.57	9.29	14.7	8.03	10.43	11.82	12.89	8.02	14	14	4	5	10	26	36
R60994	CM	M	74.72	160	25.97	17.65	10.72	17.8	18.84	25.66	13.09	32.58	5.75	8.5	13.51	7.39	9.61	8.57	11.34	8.14	13	15	4	5	12	27	38
R44217	CM	M	72.15	134	23.72	17.43	10.57	17.8	20.63	24.55	13.4	29.39	5.27	8.14	13.17	6.84	11.26	9.42	11.71	5.81	13	13	4	4	10	21	40
R41167	CM	M	77.14	153	25.93	17.23	11.39	19.81	21.38	25.41	15.22	28.58	5.15	9.04	13.95	8.54	12.72	11.17	12.24	5.99	15	14	5	4	11	24	37
R41630	CM	M	73.55	137	25.03	17.86	10.15	20.96	22.15	26.02	13.55	29.99	5.86	8.7	11.95	7.36	12.09	10.95	13.46	8.03	14	14	5	5	12	25	36
R34386	CM	M	79.83	152	27.24	18.46	14.23	17.58	20.09	24.13	11.83	31.97	6.82	8.68	16.45	6.91	11.28	9.35	9.84	6.68	15	15	4	5	10	23	40

R41631	CM	M	76.5	NA	26.33	18.49	13.09	19.2	21.96	25.59	14.25	31.17	5.84	8.81	13.42	8.38	13.88	10.57	12.86	7.56	15	14	5	5	11	22	38
R53106	CM	M	72.32	137	24.02	16.61	10.94	18.35	19.19	26.8	15.38	29.64	5.4	8.73	12.56	6.98	13.25	10.76	12.28	6.71	16	16	5	5	12	24	39
R52910	CM	M	77.67	149	26.55	19.21	11.44	20.36	22.94	30.77	17.23	27.95	6.21	9.07	13.52	8.03	9.83	9.96	14.68	8.26	14	14	5	5	11	26	40
R40692	CM	M	75.79	133	26.11	18.55	11.49	18.22	21.54	27.45	14.46	28.49	5.84	9.41	13.82	7.82	10.93	10.57	10.83	7.2	15	16	5	6	11	24	39
R40693	CM	M	75.33	137	28.68	18.61	12	18.75	21.54	24.25	12.55	32.76	5.49	9.42	14.44	8.17	10.15	11.15	11.42	6.82	16	17	5	5	10	24	37
R40690	CM	M	73.44	128	26.02	18.97	10.51	19.19	20.81	25.35	14.24	33.48	5.64	8.36	14.44	7.92	10.69	11.96	12.58	7.71	15	16	5	6	12	27	39
R13797	CM	M	73.99	126	26.07	18.85	11.59	18.86	19.73	24.04	15.41	31.68	6.08	8.9	14.36	8.29	11.32	9.02	13.85	7.98	15	15	4	4	10	19	44
R65298	CM	M	69.13	123	22.96	16.82	9.59	18.05	15.4	22.98	12.86	27.21	5.74	8.11	11.15	6.2	10.84	10.21	13.48	8.29	17	17	5	5	10	25	34
R46292	CM	M	76.62	157	27.84	19.16	10.86	19.06	20.46	25.96	14.13	26.84	6.03	9.48	14.64	7.14	10.75	10.31	13.92	7.82	16	17	5	6	12	24	42
R62521	CM	M	76.35	147.71	24.92	17.87	10.53	19.25	19.28	26.49	14.19	34.81	4.86	8.95	13.46	7.71	9.5	10.31	11.71	7.93	17	18	5	6	12	25	38
R51819	CM	M	78.2	156	26.69	18.51	12.34	21.19	24.96	27.65	15.37	27.23	5.45	9.68	14.35	7.53	12.32	9.38	13.39	7.91	14	14	5	5	12	24	40
R57125	CM	M	78.99	NA	25.83	18.46	12.09	18.85	20.6	28.01	13.73	33.22	5.8	8.1	14.5	7.73	14.66	10.42	11.55	8.66	16	16	4	5	12	23	41
R52169	CM	M	68.49	146	24.37	17.39	10.29	20.01	21.57	28.76	16.77	26.33	4.95	8.95	12.35	7.73	9.89	9.46	14.23	8.99	16	15	5	5	12	24	43
R41208	CM	M	77.78	NA	27.89	19.69	12.09	20.57	23.09	29.87	16.94	26.78	5.99	9.01	14.13	8.35	13.58	11.06	13.74	7.92	16	17	5	6	11	25	38
R41637	CM	M	74.93	126	25.85	19.03	10.81	17.63	20.21	24.44	13.73	28.32	5.63	9	14.76	8.39	10.98	10.84	13.91	8.06	16	16	5	5	12	22	41

R58317	CM	M	70.61	140	23.95	17.03	10.55	16.87	16.4	26.7	15.24	27.46	6.24	8.45	12.68	6.86	10.79	8.44	14.09	8.57	13	14	4	6	11	25	35
R65475	CM	M	68.42	126	24.12	16	10.01	19.19	17.32	24.47	14.15	29.9	5.65	7.71	13.63	6.6	11.79	9.79	13.18	7.67	15	17	4	5	12	26	44
R63934	CM	M	72.6	126	27.23	19.16	10.64	19.15	18.75	23.65	13.45	27.1	5.54	9.33	14.19	7.18	12.41	8.99	13.84	7.55	17	18	4	5	12	24	42
R52283	CM	M	77.19	146	26.76	19.71	11.35	20.59	21.25	27.36	15.02	28.55	4.92	9.19	14.68	8.18	12.86	11.47	14.74	8.86	17	14	4	5	11	25	42
R53234	CM	M	83.78	NA	29.24	20.14	12.64	19.74	20.19	26.24	14.97	33.87	5.54	9.96	15.54	8.41	12.76	10.79	12.02	6.43	15	15	5	6	12	27	44
R53217	CM	M	78.27	NA	27.1	18.6	12.66	20.75	22.27	28.96	16.5	30.94	5.1	9.49	14.44	8.05	13.2	12.01	15.56	8.62	16	18	5	6	12	25	39
R51757	CM	M	70.07	161	25.61	16.86	11.15	19.69	22.58	29.09	17.07	26.1	5.81	8.79	12.17	7.31	11.75	10.41	14.82	9.48	15	15	5	5	11	27	36
R57152	CM	M	76.15	143	27.63	19.79	12.47	20.13	21.79	28.6	16.5	30.83	5.74	9.88	13.54	7.72	12.09	10.73	14.08	8.38	16	15	6	5	12	24	36
R57149	CM	M	78.88	136	26.62	18.9	11.54	19.92	18.91	25.33	15.12	34.88	6.13	8.52	15.35	8.34	10.64	10.75	13.01	8.64	15	16	4	5	11	26	43
R57140	CM	M	76.2	143	26.48	19.59	12.1	19.6	20.49	27.82	15.9	32.69	5.34	8.69	14.07	8.12	10.12	11.65	13.83	8.74	15	15	4	5	11	26	40
R57146	CM	M	76.31	159	26.85	17.93	10.72	21.88	21.9	27.24	17.15	31.75	5.83	9.98	14.39	7.77	13.8	11.64	13.82	8.79	16	16	5	5	11	24	36
R57144	CM	M	83.12	166	30.49	20.57	13.53	22.2	20.51	28.68	18.4	35.05	6	9.15	15.38	7.95	12.52	12.73	14.91	9.18	18	17	5	5	10	25	42
R57143	CM	M	84.62	NA	29.29	19.62	12.05	19.27	20.65	28.79	15.13	32.73	6.2	11.02	13.83	8.29	11	10.83	12.54	8.33	17	17	5	6	12	26	42
R57147	CM	M	75.36	154	27.03	18.31	12.77	21.13	21.6	27.52	15.6	33.86	6.05	9.45	13.3	7.86	11.38	11.24	12.91	7.33	17	16	5	5	11	23	41
R57151	CM	M	79.63	153	28.84	20.77	13.94	21.55	20.07	26.69	13.64	32.08	5.98	9.73	15.8	8.47	10.1	10.66	13.96	8.1	16	16	4	5	12	22	36
R57141	CM	M	74.44	174	25.92	18.14	10.92	18.58	20.71	28.17	16.97	30.02	5.32	8.41	13.83	7.23	12.68	10.84	14	8.28	16	16	5	6	12	26	43

R57145	CM	M	79.12	154	27.15	18.55	11.34	18.48	19.55	27.94	17.28	33.24	5.93	9.33	14.29	7.8	12.15	10.66	13.72	8.13	16	16	4	6	12	24	40
R57142	CM	M	84.3	169	29.34	19.69	11.83	20.7	21.95	27.82	16.51	36.22	5.87	9.56	15.11	8.44	11.26	11.05	12.49	9.26	17	16	5	6	12	26	40
R57148	CM	M	70.9	132	26.3	17.81	11.46	19.55	20.48	28.29	17.81	28.54	5.8	8.72	13.21	8.36	10.23	9.61	13.39	7.96	16	16	5	6	12	26	41
R57139	CM	M	78.95	151	26.79	19.2	12.37	21.84	21.56	29.77	17.17	30.13	5.81	8.86	14.05	8.02	9	11.27	14	8.56	15	15	4	5	12	24	40
R57150	CM	M	79.91	152	29.72	20.56	12.6	18.52	18.35	24.42	14.52	33.15	6.18	10.4	15.26	8.49	11.24	11.09	12.66	7.2	16	16	5	6	11	25	34
ZMB54516	CM	M	52.9	95.6	18.1	14	7.8	14.5	17.4	21.3	11.5	24.3	3.8	6.6	8.3	5.3	7.7	8.5	11.7	5.6	16	19	5	4	12	25	42
NMVD3413	CD	M	67.48	128	22.29	16.03	8.61	14.38	15.06	24.53	13.91	26.69	4.68	8.28	11.4	5.33	11.07	9.48	11	6.77	16	16	4	6	13	25	45
R54725A	CD	M	66.03	136	24.42	16.99	9.5	17.85	18.97	23.8	14.59	24.48	5.51	7.47	11.82	6.82	12.18	9.28	12.58	7.54	16	15	5	5	12	25	40
R54725B	CD	M	66.28	137	24.06	16.84	9.5	17.15	18.76	24.99	14.56	24.58	5.25	8.06	11.92	6.68	11.55	9.43	12.74	7.67	15	15	5	5	11	27	40
R54926	CD	M	67.8	NA	24.06	16.83	9.47	16.32	18.84	25.92	14.8	21.65	5.1	8.39	14.25	6.89	11.54	9.93	11.21	7.49	16	15	5	5	12	25	41
R23528	CD	M	74.17	154	24.58	16.93	9.8	18.19	20.83	27.78	15.18	32.68	4.73	8.92	12.24	7.5	11.21	10.5	13.01	6.35	15	14	4	5	12	26	45
R13517B	CD	M	65.2	129	23.47	16.04	9.83	16.07	18.16	25.65	14	25.39	5.51	8.12	12.38	5.62	9.75	8.34	13.15	8.99	14	14	4	5	12	26	41
R62501	CD	M	71.97	151	24.38	18.51	9.57	17.5	16.57	26.36	13.67	27.5	5.22	8.89	12.71	7.47	9.55	10.05	12.42	7.03	14	14	5	5	12	26	41
R81663	CD	M	74.54	NA	24.36	17.56	12.96	19.12	19.49	27.04	14.73	33.58	5.38	8.89	11.41	7.34	10.2	9.91	13.46	7.78	12	12	4	4	10	29	41
R81664	CD	M	75.71	156	24.64	16.71	11.69	19.92	18.11	26.56	15.24	28.34	5.46	9.02	12.58	7.52	11.46	9.72	13.49	7.43	14	13	4	5	11	24	43
R32773	CD	M	67.84	NA	24.23	17.76	10.67	17.05	19.82	26.49	15.47	30.58	5.68	8.81	11.9	7.34	9.42	9.67	11.58	6.21	14	15	5	5	12	24	46

R55110	CD	M	70.37	NA	25.06	16.08	9.46	17.31	18.32	23	13.22	28.77	5.91	8.31	12.34	7.03	11.34	9.8	11.83	6.69	14	14	4	5	11	26	42
R55109	CD	M	73.82	NA	25.77	17.69	9.58	16.4	18.27	26.65	15.29	31.64	5.49	8.99	13.61	7.4	11.21	9.83	11.9	6.67	14	13	5	5	11	25	46
R54830	CD	M	66.4	126	22.57	15.31	8.67	19.55	20.33	27.69	16.98	25.53	5.46	7.65	11.62	6.13	12.46	9.76	12.88	8.19	14	15	5	6	11	24	43
R12075	CD	M	70.19	134	25.08	17.28	11.05	16.89	19.53	27.11	14.66	28.71	5.16	8.38	12	6.95	10.12	8.55	12.08	8.34	14	14	5	5	11	23	40
R37486	CD	M	68.58	150	23.76	17.18	9.73	18.51	19.29	26.75	15.89	24.03	5.54	7.88	12.07	7.14	10.23	9.11	12.4	6.85	15	14	5	4	11	25	42
R81665	CD	M	77.53	144	26.07	17.16	11.94	16.35	18.28	26.66	16.03	33.25	5.23	9.05	12.79	7.56	9.47	9.31	12.15	7.53	14	15	5	5	11	25	45
R83249	CD	M	77.44	148	24.73	17.59	11.58	16.6	21.01	27.94	16.71	31.37	5.18	9.03	13.03	6.73	9.45	10.15	13.79	8.14	15	15	5	5	13	29	42
R81640	CD	M	70.63	135	21.71	15.37	9.9	18.84	20.66	28.62	16.48	28.66	5.53	7.51	10.7	6.83	10.78	10.82	14.66	8.93	15	14	5	5	11	26	44
R53691	CD	M	68.29	127	23.69	16.23	9.86	17.76	18.92	23.54	13.65	28.06	5.17	7.4	13.03	6.54	10.33	9.7	10.83	7.71	14	14	4	5	11	26	40
R11358	CD	M	69.67	NA	24.9	18.18	10.9	17.26	18.41	24.34	13.82	29.24	5.3	8.96	13.17	7.14	12.59	8.8	11.97	8.04	14	13	4	5	12	25	43
R11359	CD	M	71.4	139	25.37	17.91	10.83	16.42	19.28	24	12.31	27.94	5.59	8.77	12.88	6.92	9.51	9.31	12.54	7.09	14	13	4	5	11	23	43
MNHN6545	CD	M	80	147.5	25	19.5	11.5	17.6	21.3	30.1	16.5	37	4.7	9.3	13.5	6.5	14	11.5	14	10.5	15	18	5	5	11	30	45
R61808	CF	F	71.87	NA	23.08	15.78	8.98	18.58	20.03	25.84	14.27	30.54	5.43	8.4	11.05	6	8.67	10.66	12.95	6.88	15	14	4	6	11	24	NA
R62709	CF	F	74.02	NA	23.94	16.81	10.28	17.92	19.8	23.38	12.38	34.42	5.34	8.62	13.04	6.78	8.87	8.62	12.35	7.62	16	16	5	7	11	22	NA
R61590	CF	F	79.6	142	25.98	17.75	9.78	19.24	21.28	26.37	14.3	36.01	5.94	9.12	12.21	7.64	11.03	10.48	14.01	7.27	13	14	4	5	10	26	NA
R12929A	CF	F	69.12	131	22.32	14.81	9.24	17.08	19.11	25.03	12.95	32.64	4.68	7.46	10.74	6.48	11.28	8.95	12.01	7.67	15	16	5	5	11	22	NA

R12929B	CF	F	68.6	131	21.39	14.81	10.18	16.78	19.73	23.74	13.34	31.43	4.92	7.58	10.88	7.01	9.91	10.04	10.97	7.47	12	14	4	5	10	25	NA
R51870	CV	F	68.48	146	21.49	15.55	9.29	16.95	21.22	24.82	14.9	33.48	5.18	7.43	10.33	6.69	11.09	9.98	12.53	7.29	15	15	4	5	11	25	NA
R51910	CV	F	70.9	NA	21.73	15.38	9.42	18.28	19.57	23.8	12.62	32.34	4.85	8.43	10.56	6.08	10.07	10.71	10.9	7.04	15	14	5	5	12	26	NA
R52213	CV	F	72.51	140	23.25	16.46	10.33	19.15	20.62	24.77	12.95	32.73	5.06	8.01	11.8	6.93	11.96	10.28	12.95	8.14	16	16	6	6	11	26	NA
R64523	CV	F	65.85	138	21.94	16.11	9.36	17.46	19.88	25.99	15.61	29.06	5.37	8.24	10.8	6.56	9.68	10.61	13.1	8.57	17	16	5	5	11	25	NA
R64632	CV	F	64.62	139	22.31	14.45	8.86	17.25	18.94	23.77	13.12	25.77	5.37	7.41	11.03	6.13	10.32	9.82	11.99	6.43	15	13	5	5	10	24	NA
NMVD3414	CM	F	73.2	NA	23.92	16.25	9.84	17.97	18.31	23.79	12.67	34.55	5.55	8.66	12.25	6.79	11.56	9.3	11.45	6.81	16	16	5	6	13	25	NA
NMVD13399	CM	F	82.93	153	24.03	17.07	10.47	19.97	20.06	25.93	11.82	37.26	5.13	9.03	14.35	6.6	11.79	9.45	12.15	8.38	15	15	5	5	12	23	NA
NMVD16515	CM	F	76.66	NA	22.4	15.57	9.62	16.36	16.49	26.11	15.78	35.42	4.8	8.43	11.11	5.45	12.79	10.64	13.67	10.25	17	16	5	4	10	25	NA
R44453	CM	F	66.23	134.85	24.32	15.82	11.89	16.95	20.62	26.75	14.82	29.41	5.51	8.37	12	7.47	9.59	9.96	12.28	6.85	14	15	5	5	9	23	NA
R60607	CM	F	74.84	NA	22.86	16.1	9.16	18.72	18.39	23.95	12.89	30.36	5.32	7.97	12.57	6.55	9.54	10.45	12.65	8.51	16	16	5	5	12	25	NA
R40708	CM	F	74.87	NA	21.67	15.89	9.6	16.37	17.11	21.32	12.31	36.67	5.02	7.93	11.49	7.22	8.15	9.08	10.16	5.55	15	16	4	4	11	22	NA
R53171	CM	F	69.23	129	22.21	14.69	9.26	17.71	16.9	24.36	13.35	29.9	5.03	7.44	10.49	6.64	11.3	10.87	12.29	7.77	15	15	4	5	11	24	NA
R53112	CM	F	72.52	131	23.56	16.14	9.97	18.07	18.78	25.62	13.24	31.93	5.04	8.8	10.94	7.54	11.77	10.86	13.87	9.07	16	15	5	5	12	27	NA
R53001	CM	F	70.36	NA	21.1	15.3	9.74	15.77	18.58	26.16	14.41	31.61	4.73	7.75	10.73	7.17	13.46	10.08	14.22	9.16	15	15	5	5	11	25	NA

R52892	CM	F	70.34	144	21.58	14.84	9.63	17.01	18.67	25.39	13.32	26.05	5.15	7.76	10.87	6.68	10.59	10.27	12.2	7.3	15	16	4	5	11	27	NA
R52935	CM	F	70.54	139	21.57	16.09	10.3	19.69	21.36	26.22	14.02	29.43	5.33	8.49	11.96	7.34	9.69	9.85	12.68	7.35	15	14	4	5	11	25	NA
R53083	CM	F	69.29	NA	22.66	16.24	9.29	18.49	19.34	23.06	12.95	31.01	4.89	7.7	10.4	6.79	11.69	10.59	13.04	8.16	15	15	5	5	12	27	NA
R53084	CM	F	70.78	139	22.28	15.58	9.12	17.18	18.68	22.75	13.56	30.15	5.41	8.46	10.6	7.23	9.63	9.6	13.8	9.46	15	16	5	5	12	25	NA
R62522	CM	F	79.48	152	24.46	16.71	10.56	18.9	17.88	25.36	13.88	35.28	5.79	8.74	11.49	7.42	10.75	11.53	14.22	9.42	17	18	5	5	11	26	NA
R52171	CM	F	75.19	NA	24.3	16.81	10.26	19.31	21.18	25.64	12.65	33.03	5.48	8.74	11.91	6.81	10.14	10.42	14.75	8.39	14	14	4	5	12	24	NA
R12910	CM	F	74.6	143	22.91	16.4	9.8	18.07	20.33	26.75	12.73	34.43	4.63	8.58	11.97	7.15	11.77	9.45	11.58	6.83	15	15	4	5	12	21	NA
R11357	CM	F	69.43	120	22.55	16.09	10.08	16.05	19.34	23.78	11.9	30.17	4.76	7.71	12.07	6.02	11.93	8.76	11.62	7.6	16	16	5	5	11	25	NA
R41638	CM	F	71	130	22.71	15.75	9.46	17.27	18.51	21.89	12.21	32.7	5.31	7.83	10.95	6.57	9.7	8.7	12.67	8.85	17	16	5	5	11	23	NA
R53269	CM	F	73.11	128	23.44	16.49	11.05	17.21	18.38	25.27	14.21	34.18	5.4	8.53	12.39	7.09	11.5	10.55	13.6	8.74	14	14	4	5	11	24	NA
R53260	CM	F	67.83	140	21.95	14.72	9.71	16.82	18.04	24.93	14.92	31.04	5.4	7.48	11.11	6.58	11.64	10.97	14	9.11	15	16	5	4	11	24	NA
R41193	CM	F	70.8	110	22.1	14.37	9.39	15.24	16.58	22.48	13.18	35.76	4.97	7.01	10.7	6.65	9.35	8.75	11.93	7.05	13	15	4	4	10	24	NA
R51988	CM	F	76.35	151	23.95	16.67	10.15	18.12	18.86	26.6	15.22	33.79	5.55	7.98	13.06	7.41	10.93	10.39	11.05	7.85	16	15	4	5	11	27	NA
R44221	CM	F	78.09	145	25.24	16.72	10.94	19.68	21.1	26.52	14.84	36.93	5.54	9.38	12.46	7.56	11.47	11.28	14.15	8.66	15	16	4	5	13	24	NA
R53225	CM	F	78.98	144	24.49	16.5	10.71	17.53	21	26.54	15.94	32.39	5.92	8.82	11.88	7.61	13.03	10.82	14.73	9.06	17	17	4	5	11	26	NA
R58326	CM	F	65.14	123	20.99	14.86	8.88	15.74	15.09	21.48	11.46	27.59	5.43	7.76	9.7	6.82	8.42	8.65	10.81	6.95	15	15	4	6	12	22	NA

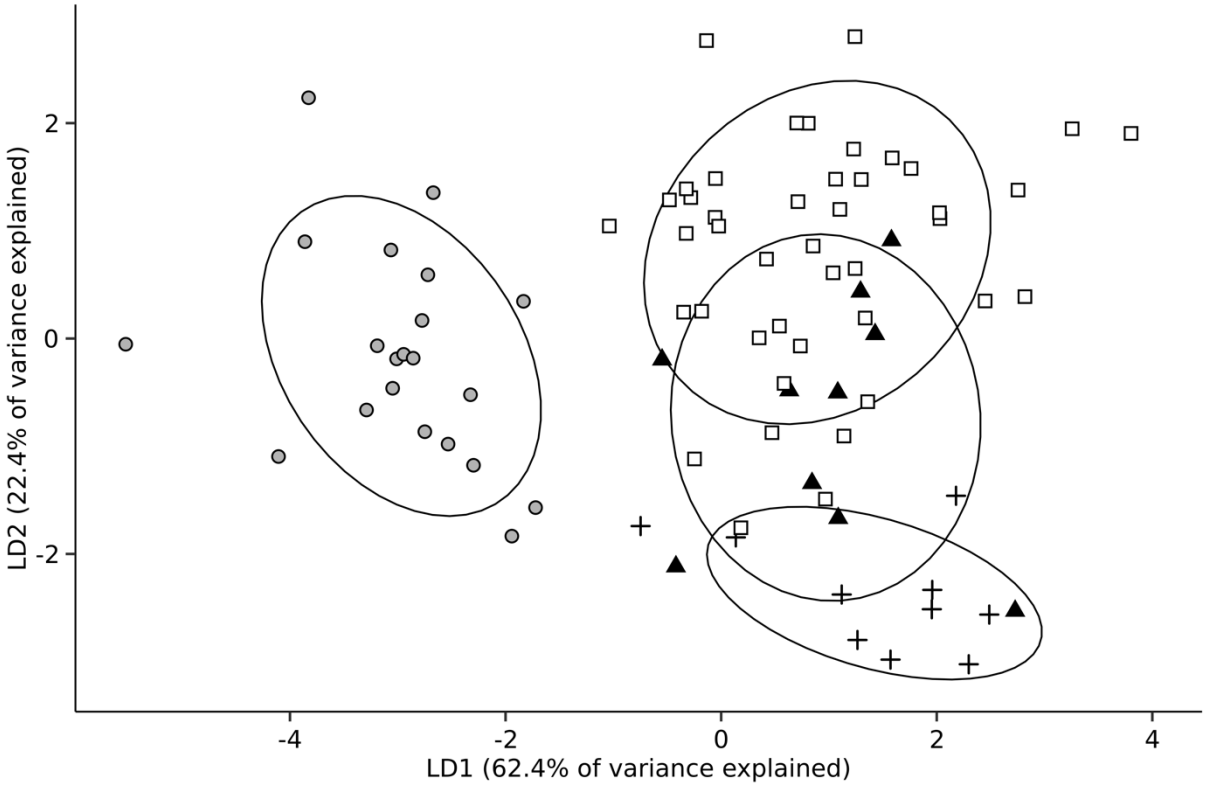
ZMB30092	CM	F	78.3	122	22.1	16.5	10.5	17.8	18.6	24	13.6	38.4	4.5	9.4	11.3	6.9	11	10.6	13.1	8	14	14	5	6	9	23	NA
R92117	CD	F	71.65	139	21.33	14.85	8.99	17.58	16.66	21.74	11.17	33.74	4.78	7.6	10.93	6.46	10.35	8.05	11.89	7.13	13	13	4	5	10	24	NA
R43155	CD	F	65.23	NA	20.56	14.08	8.03	16.03	18.15	23.3	12.75	30.61	4.73	7.07	10.42	6.06	10.5	9.18	11.25	7.17	15	15	4	5	11	26	NA
R13968	CD	F	70.12	123	21.23	15.89	10.21	16.11	17.96	22.45	11.79	29.96	5.91	7.61	10.38	6.48	8.05	8.63	11.25	7.04	16	16	4	5	11	24	NA
R55041	CD	F	65.87	125	20.33	14.42	8.33	14.95	16.78	23.5	13.11	28	4.54	7.15	10.44	6.37	9.59	8.36	12.47	8.6	14	14	5	5	12	26	NA
R54876	CD	F	65.65	NA	21.26	15.14	8.89	16.79	18.69	23.58	12.12	27.61	5.23	7.22	10.36	6.5	11.72	10.46	10.68	6.88	14	13	4	5	10	26	NA
R81662	CD	F	69.82	NA	22.32	14.83	10.02	17.97	19.39	24.62	12.88	30.42	5.02	7.54	9.76	6.87	9.98	9.81	11.7	8.22	14	13	4	5	11	26	NA
R83248	CD	F	67.93	128	20.6	14.39	9.13	17.18	17.8	23.76	13.45	31.91	5.03	6.89	10.05	6.47	10.23	9.68	12.02	7.17	13	11	4	5	12	25	NA
R81637	CD	F	69.35	133	20.47	15.2	9.84	16.78	18.05	23.36	12.67	29.29	5.41	7.15	10.71	6.59	9.22	9.58	13.47	8.23	12	12	4	4	11	22	NA
R32772	CD	F	68.43	NA	23.09	15.65	10.19	15.98	19.33	22.52	13.49	29.98	5.36	7.82	10.9	6.59	8.88	8.87	10.13	5.7	14	15	4	5	12	24	NA
R32778	CD	F	66.69	NA	21.55	14.79	9.85	14.28	16.88	22.6	11.69	29.39	4.98	7.59	10.41	6.64	10.27	9.03	10.42	6.54	15	14	4	5	13	21	NA
R32777	CD	F	62.05	NA	19.81	13.27	9.01	15.53	18.5	25.52	15.02	26.63	4.68	6.81	9.48	6.12	16.17	17.88	22.53	13.05	14	14	4	5	11	21	NA
R57376	CD	F	73.08	132	23.38	16.48	9.28	15.83	17.69	23.28	12.89	33.74	5.08	8.12	12.44	7.61	8.76	8.8	13.81	8.2	16	15	5	6	12	27	NA
R54887	CD	F	61.86	124	21.49	14.6	8.51	14.28	16.38	23.68	13.05	25.03	4.54	7.41	10.37	6.32	10.76	9.29	12.63	7.78	15	15	5	5	11	24	NA
R42978	CD	F	74.13	NA	22.97	15.5	8.35	16.68	19.37	23.45	13.05	31.77	5.21	8	10.9	5.95	9.31	10.33	13.02	8.05	18	14	5	6	13	25	NA

R55108	CD	F	71.69	143	22.39	15.52	8.66	17.08	19.19	23.97	14.14	31.89	5.55	8.4	11.83	7.36	12.46	10.36	12.4	8.38	15	15	6	5	12	25	NA
R81639	CD	F	68.28	129	20.94	14.68	10.02	17.95	18.24	25.41	14.67	31.21	4.56	7.89	10.6	6.64	9.75	9.39	12.74	7.22	13	14	5	5	11	26	NA
R81638	CD	F	66.62	NA	20.12	14.07	9.21	16.96	18	24.26	13.85	29.64	4.6	7.73	9.65	5.91	8.84	9.64	12.6	7.12	13	13	4	5	11	23	NA
R53785	CD	F	72.18	126	23.27	16.64	10.47	17.45	19.21	24.57	13.43	32.44	4.97	8.43	12.42	6.88	9.09	10.34	12.51	7.38	17	14	5	5	11	22	NA
R45128	CD	F	71.8	129	22.33	15.42	8.81	16.44	18.52	24.28	12.84	31.72	4.86	8.88	11.29	6.68	9.89	9.16	13	7.41	14	14	5	4	11	21	NA
MNHN6545A	D	F	62.5	119	20.5	15.5	9	15.5	17.2	26.6	15.2	29	4.65	7.6	10.5	6.3	11.5	10	12.9	10	15	16	5	6	11	27	NA

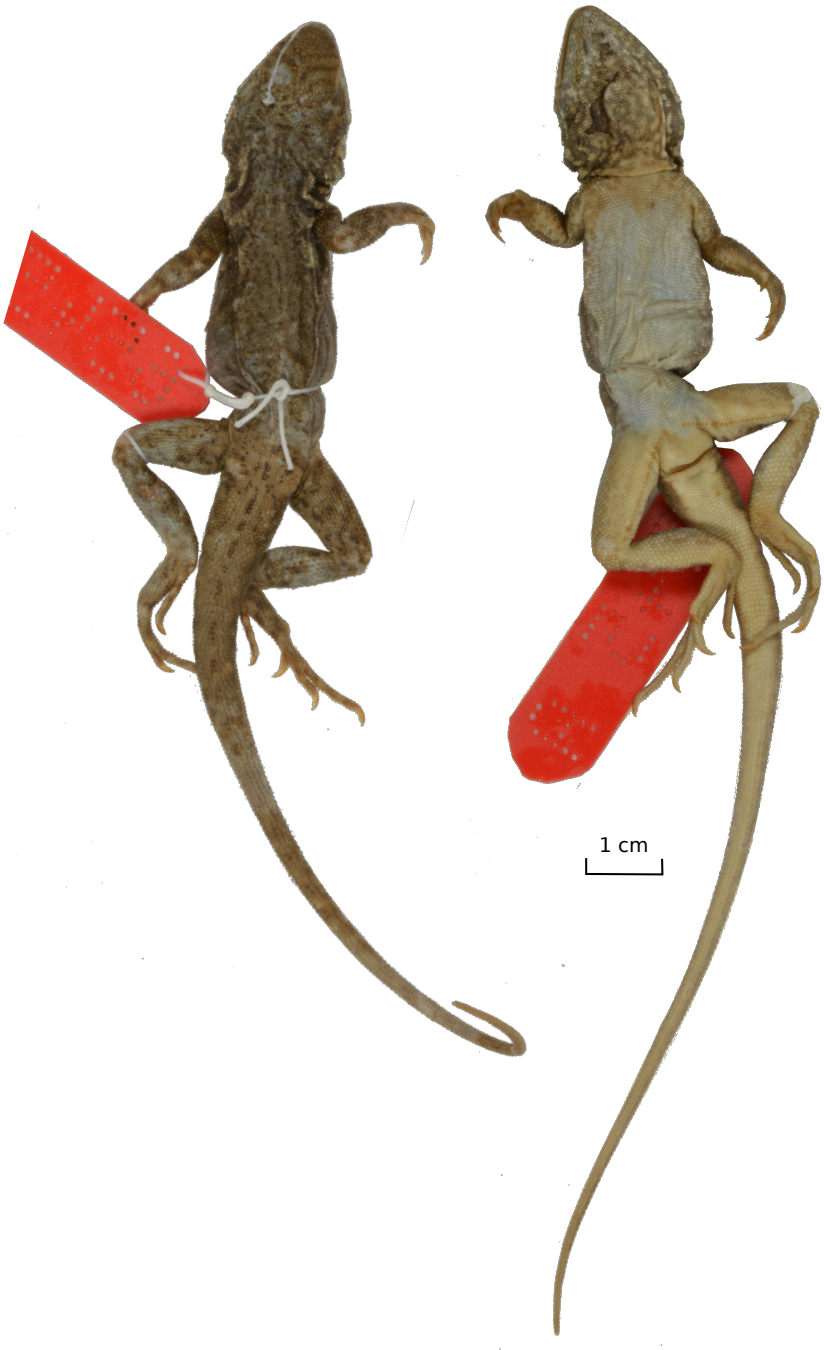
Appendix 5.2. Standardised linear discriminant coefficients for the first two canonical variables for the analysis of males with 24 variables. Characters strongly correlated with canonical variables (large absolute values) are bolded and italicised.

Character	LD1	LD2
SVL	***1.183***	-0.147
AGL_SVL	0.471	-0.060
HL_SVL	***-0.799***	0.411
HW_SVL	0.378	-0.409
HD_SVL	-0.121	0.374
EYE_SVL	0.189	-0.037
SL_SVL	0.022	-0.254
JL_SVL	0.633	-0.338
NW_SVL	0.249	0.367
HUML_SVL	0.133	0.106
RADL_SVL	-0.483	-0.007
HANL_SVL	0.666	0.198
FINL_SVL	-0.295	0.285
FEML_SVL	0.229	-0.169
TIBL_SVL	***0.795***	***-0.659***
FOOT_SVL	0.316	***-0.682***
TOE_SVL	-0.290	0.068
SUPRA	0.165	-0.110
INFRA	***0.866***	0.440
ROSNAS	-0.214	0.219
SUPRANAS	0.067	-0.081
INTERNAS	-0.183	0.270
SDL	-0.177	-0.102
FP	***-0.733***	-0.218
Proportion of Variance	0.624	0.224

Appendix 5.3. Linear discriminant analysis of males based on 24 variables (including snout-vent-length): *Ctenophorus modestus* (□); *C. decresii* sensu stricto (●); *C. fionni* (▲); and *C. vadnappa* (+). 95% confidence ellipses are shown.



Appendix 5.4. Dorsal (left) and ventral (right) photographs of the paralectotype of *Ctenophorus decresii* (Duméril and Bibron, 1837; MNHN 6545A). This specimen is an adult female collected from Kangaroo Island, South Australia, in January 1803 (photos Nicolas Vidal, Muséum national d'Histoire naturelle Paris).



Appendix 5.5. Dorsal (left) and ventral (right) photographs of the paralectotype of *Ctenophorus modestus* (Ahl, 1926; ZMB 30092). This specimen is an adult female from "Australien" without an exact locality or reference to a collector or donor (photos Frank Tillack, Museum für Naturkunde Berlin).



Chapter 6: General discussion

In this thesis, I explored aspects of divergence and the evolutionary consequences of secondary contact between a colour polymorphic northern lineage and monomorphic southern lineage of the tawny dragon lizard, *Ctenophorus decresii* sensu lato, with particular focus on the role of sexual colour signals. Here I discuss key findings, review knowledge gaps, and suggest potential avenues for future research.

6.1 INTRINSIC INCOMPATIBILITIES DURING SECONDARY CONTACT

My work has ultimately resulted in the elevation of the lineages of *C. decresii* sensu lato into separate species (Chapter 5): *C. decresii* sensu stricto (previously southern lineage) and *C. modestus* (previously northern lineage). The two lineages have long been regarded as distinct on the basis of male coloration variation (Houston 1974a) and more recently, genetic divergence (McLean et al. 2014b). Through a detailed investigation of the contact zone between the lineages, I demonstrated that gene flow is restricted and hybridisation is not ongoing, providing justification for their taxonomic separation. Lineage diversification in *C. decresii* sensu lato was initiated by climatic oscillations of Pleistocene glacial-interglacial cycles and ensuing contraction to allopatric refugia (Byrne 2008; McLean et al. 2014b). During isolation, lineages would have accumulated a series of genetic changes, either adaptive or non-adaptive (genetic drift), on the pathway to reproductive isolation. This origin is consistent with phylogeographic patterns found in many other southern Australia species (see references in Byrne 2008).

During secondary contact, genetic incompatibilities primarily restrict gene flow between *C. decresii* sensu stricto and *C. modestus* (Chapter 3). The contact zone is comprised of post-F1 hybrids with *C. modestus* mtDNA, which asymmetrically backcross to *C. modestus* and virtually

do not backcross to *C. decresii* sensu stricto. I identified a narrow density trough (ca. 12 km) across which the hybrid index (genomic Bayesian q-value) and mtDNA haplotype compositions change abruptly. The density trough was surveyed thoroughly in 2016 and 2017, and briefly in 2013. The rocky habitat from the contact zone to the Mount Lofty populations is continuous; only one minor road exists in this stretch and is not expected to hinder movement. The absence of obvious geographical barriers to dispersal suggests that intrinsic incompatibilities prevent backcrossing to *C. decresii* sensu stricto, rather than extrinsic factors. A lack of extrinsic factors bounding hybridisation would open the possibility of a moving contact zone, where the geographical location of hybridisation shifts over time, potentially affecting patterns of introgression. Many intrinsic and extrinsic factors can contribute to moving contact zones (see references in Billerman et al. 2019), including climate change (Buggs 2007; Taylor et al. 2015). In this case, the contact zone may move further south in response to climate change, potentially infringing upon the range of *C. decresii* sensu stricto. Ongoing surveys would be necessary to detect contact zone movement.

Through captive breeding experiments, I found differences in maternal reproductive strategy and investment between species and disparities in reciprocal hybrid fitness, which may contribute to genetic patterns observed in the contact zone (Chapter 4). Females of *C. modestus* had higher overall reproductive output and produced F1 hybrid offspring which exhibited higher trait values for more fitness measures relative to the reciprocal cross. These factors together would likely inundate the contact zone with *C. modestus* mtDNA during the F1 and successive generations, consistent with findings from in the field (Chapter 3). Furthermore, the reduced fitness of hybrid crosses (in this case F1 hybrids sired by *C. modestus* males) would be expected to select for premating barriers to prevent maladaptive hybridisation. Behavioural experiments in captivity indicate weak behavioural barriers to pairings between genetically pure populations of *C. decresii* sensu stricto males and *C. modestus*

females (McLean et al. 2020; Appendix 6.5). Specifically, *C. decresii* sensu stricto males are more likely to court and attempt copulation with females of their own species and be rejected by females of *C. modestus*. By contrast, there is no evidence for behavioural barriers to mating between *C. decresii* sensu stricto females and *C. modestus* males, which is opposite to predictions derived from the data on F1 offspring fitness as well as genetic patterns in the contact zone. However, the degree of pre-mating isolation between allopatric populations is often not indicative of mating patterns within contact zones (sympatry) because pre-mating isolation may be enhanced in sympatry due to reinforcement (Servedio and Noor 2003; Coyne and Orr 2004). Therefore, within the contact zone, we would expect pre-mating barriers to prevent maladaptive pairings between *C. modestus* males and *C. decresii* sensu stricto females given evidence of genetic incompatibility and fitness costs. The strength of reinforcement in the contact zone has not been directly assessed, although there is preliminary field-based evidence that hybrid males do not show a biased territorial aggressive response towards intruders from either parental species (Mesis 2016). Research examining reinforcement within the contact zone would be valuable to fully understand mechanisms which have driven speciation to completion between *C. decresii* sensu stricto and *C. modestus*.

In theory, divergent sexual selection alone can drive pre-mating isolation and speciation (Ritchie 2007; Maan and Seehausen 2011; Scordato et al. 2014; Servedio and Boughman 2017). However, in this system sexual signal divergence (male throat coloration) does not correspond with divergence in mate preferences (McLean et al. 2020; Appendix 6.5). Additionally, there is no evidence that the two species differ in male competitive ability, suggesting that intra-sexual selection is unlikely to influence the outcome of secondary contact (McLean et al. 2020; Appendix 6.5). Rather, speciation is ultimately driven by genetic incompatibility, possibly together with pre-mating barriers which evolved in sympatry via reinforcement, whereas pre-mating behavioural barriers in allopatric populations are

incomplete. These findings are consistent with the emerging view that speciation is rarely driven by sexual selection alone, but acts in conjunction with natural selection (Servedio and Boughman 2017).

6.2 ROLE OF THROAT COLORATION IN DIVERGENCE

Sensory drive is predicted to drive speciation between allopatric populations following secondary contact, due to the key role of differing habitat properties (physical and structural) in driving mating trait divergence. In this system, despite the adaptive divergence of mating traits (sexual colour signals; McLean et al. 2014a) between species, I found no evidence of a correlated divergence in visual sensitivities (Chapter 2). Thus, sensory drive does not appear to be a mechanism that has contributed to the early stages of reproductive isolation (mate preferences). This is likely attributable to the rarity of female mate choice in lizards (Lebas and Marshall 2001; Olsson and Madsen 1995; Smith and Zucker 1997; Tokarz 1995). Further, terrestrial light environments vary too greatly in time and space weakening the strength of selection on visual systems (Lind 2017). Alternatively, males may select backgrounds with spectral properties that maximise conspicuousness of their throats to conspecifics.

I found evidence of selection for the *C. modestus* polymorphism within the contact zone, with low UV reflectance and all four morphs present, whereas the throat phenotypes of F1 hybrid males were highly variable, particularly in UV reflectance (Chapter 3). Future work using visual modelling to evaluate the conspicuousness of different hybrid throat phenotypes within the contact zone habitat may provide further insight into F1 hybrid fitness. This would clarify whether microhabitat characteristics of the contact zone contribute to selection against UV reflectance or if it is driven by other factors. The similarity of the contact zone habitat to that of *C. modestus* indicates that parental *C. modestus* phenotypes would be

advantageous relative to hybrid phenotypes. However, the persistence of hybrids (and absence of pure *C. modestus* individuals) may be facilitated by adaptive behavioural mechanisms to increase conspicuousness via background selection. However, there was no evidence of selection against minor leakage of the *C. decresii* sensu stricto blue coloration into the hybrid throat phenotype, as it occurred comparably in both captive-bred F1 and post-F1 hybrids. This points towards an uncoupling of the blue coloration and UV reflectance found in *C. decresii* sensu stricto, with UV reflectance being a stronger sexual signal.

Maintenance of the colour polymorphism in the contact zone, despite gene flow from monomorphic *C. decresii* sensu stricto, is consistent with predictions based on the genetic architecture of polymorphism. This stems from the existence of co-adapted trait complexes of morphs, which may be underpinned by either tight physical linkages between genes or genetic correlation between unlinked genes maintained by linkage disequilibrium and natural selection (Sinervo and Svensson 2002; McKinnon and Pierotti 2010). Further work could focus on investigating genomic signatures of speciation between these genetically and phenotypically divergent species. Given evidence that the polymorphic signal is the target of selection in the contact zone, we would expect to find corresponding evidence that loci associated with colour morphs are under selection. The detection and characterisation of loci associated with the polymorphism of *C. modestus* is an ongoing project (McLean, Moussalli, Dasmahapatra, Stuart-Fox, unpublished data). These data would allow us to test hypotheses regarding whether throat coloration is governed by few genes of major effect, as has been found in other systems, and whether these loci show greater than expected divergence between species.

6.4 CONCLUSIONS

Investigation into the outcome of secondary contact between these two genetically and phenotypically divergent species has provided insight into the importance of geographic colour variation as an indicator of incipient speciation. The outcome of gene flow between colour polymorphic and monomorphic lineages or populations was largely unexplored prior to the work in this thesis, and much still remains to be understood. The lack of previous studies may be partially because we have only recently begun to appreciate the complexity of geographic variation in polymorphism (number, type, frequencies of morphs) and its role in speciation (e.g. McLean and Stuart-Fox 2014). In addition, characterising geographic variation can be challenging, as well as the confident identification of monomorphic populations or lineages. The existence of a natural contact zone between these lineages (now species) which differ in morphs, and where genetic divergence and the biology of the polymorphism is well-characterised, provides an excellent research opportunity. Future work would be valuable to assess how broadly our conclusions generalise to other systems and taxa; for example, whether selection favours polymorphism in other contact zones, and if new morphs are generated or existing morphs are lost. This may be best undertaken in an established system with evidence of morph-specific co-adapted traits, and therefore a high likelihood of underlying genetic linkage or correlation. Further, short generation times would be useful in forming multi-generation inferences using captive studies. Such studies would build upon the foundation provided in this thesis on the microevolutionary processes linking colour polymorphism and speciation.

6.5 APPENDICES

Appendix 6.1. Divergent male and female mate preferences do not explain incipient speciation between lizard lineages.

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Divergent male and female mate preferences do not explain incipient speciation between lizard lineages

Claire A. McLean [a,b,*], Richard A. Bartle[a], Caroline M. Dong [a,b], Katrina J. Rankin[a], and Devi Stuart-Fox[a]

[a]School of BioSciences, The University of Melbourne, Parkville VIC 3010, Australia and [b]Sciences Department, Museums Victoria, Carlton VIC 3053, Australia

*Address correspondence to Claire McLean. E-mail: cmclean@museum.vic.gov.au.

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Abstract

Diversification in sexual signals is often taken as evidence for the importance of sexual selection in speciation. However, in order for sexual selection to generate reproductive isolation between populations, both signals and mate preferences must diverge together. Furthermore, assortative mating may result from multiple behavioral mechanisms, including female mate preferences, male mate preferences, and male–male competition; yet their relative contributions are rarely evaluated. Here, we explored the role of mate preferences and male competitive ability as potential barriers to gene flow between 2 divergent lineages of the tawny dragon lizard, *Ctenophorus decresii*, which differ in male throat coloration. We found stronger behavioral barriers to pairings between southern lineage males and northern lineage females than between northern males and southern females, indicating incomplete and asymmetric behavioral isolating barriers. These results were driven by both male and female mate preferences rather than lineage differences in male competitive ability. Intrasexual selection is therefore unlikely to drive the outcome of secondary contact in *C. decresii*, despite its widely acknowledged importance in lizards. Our results are consistent with the emerging view that although both male and female mate preferences can diverge alongside sexual signals, speciation is rarely driven by divergent sexual selection alone.

Key words: *Ctenophorus decresii*, hybridization, male–male competition, secondary contact, sexual selection

Populations that evolve in allopatry often diverge in sexual signals, which intuitively implicates sexual selection in speciation. However, speciation by sexual selection requires associated mating preferences in order to generate prezygotic isolation between populations should they come into contact (West-Eberhard 1983; Panhuis et al. 2001; Ritchie 2007). Both signals and preferences can diverge due to drift or local adaptation to different environments rather than, or in addition to, divergent sexual selection (Schluter 2009; Langerhans and Riesch 2013; Mendelson et al. 2016). Thus, sexual signals and mating preferences (or other behaviors involved in acquiring mates, such as male competitive ability) can evolve independently, and several outcomes are possible when populations meet. Behavioral reproductive barriers could be absent, weak, strong, or asymmetrical, resulting in varying levels of admixture, and the potential breakdown of signal and preference differences. For example, song phenotype acts as a barrier to gene flow between subspecies of white-crowned sparrows, but admixed individuals in the contact zone exhibit slightly decreased discrimination, which has the potential to weaken isolation over time (Lipshutz et al. 2019). Consequently, determining the extent to which divergent sexual signals generate premating reproductive isolation upon secondary contact is critical to understand the role of sexual selection in speciation.

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Premating isolating barriers between divergent lineages generate patterns of assortative mating. However, assortative mating can arise from multiple behavioral mechanisms such as female mate preference (or rejection of non-preferred mates), male mate preference, and competition between males for access to females. Most studies examining premating isolating barriers focus on female mate preferences but in some species, particularly those that are highly territorial and polygynous, competition between males for access to females can be the primary determinant of male reproductive success (Anderson 1994; Fitzpatrick et al. 2012; Qvarnström et al. 2012; Uller et al. 2013; McDiarmid et al. 2017; Tinghitella et al. 2018). Furthermore, female access to mates may be limited when males dominate territories encompassing the home range of one or more females and/or exhibit mate guarding behavior (Qvarnström et al. 2012). If males control mating opportunities and do not discriminate between conspecific and heterospecific females, then we may see little evidence of assortative mating. Selection for mate discrimination is expected to be weaker in males than females due to generally lower male *per capita* investment in reproduction, particularly in the absence of parental care (Bateman 1948; Anderson 1994; but see Edward and Chapman 2011). Male mate discrimination between females from divergent lineages may also be limited because females are phenotypically similar (i.e., only male sexual signals have diverged). Our understanding of the relative contributions of male–male competition and male mate discrimination in influencing the outcome of secondary contact remains limited but is of growing interest (Peterson et al. 2005; Johannesson et al. 2008; Vallin et al. 2011; While et al. 2015; Heathcote et al. 2016; Martin and Mendelson 2016).

The Australian tawny dragon lizard *Ctenophorus decresii* (Duméril and Bibron, 1837) is well suited to examining the evolution of behavioral mechanisms that may generate assortative mating upon secondary contact. This small sexually dimorphic agamid lizard comprises 2 genetically and phenotypically divergent lineages: "northern" and "southern" (Houston and Hutchinson 1998; McLean et al. 2014b; Figure 1). There is evidence that the lineages are incipient species, and the level of divergence between them (3.7% net sequence divergence in mtDNA) is consistent with contraction to, and expansion from, isolated refugia during Pleistocene glacial–interglacial cycles (Byrne 2008; McLean et al. 2014b). The lineages meet in a narrow contact zone (ca. 20 km) where F1 generation hybrids are not present (i.e., contact is not recent) and genetic admixture is asymmetrical (McLean et al. 2014b; Dong et al. 2019). Specifically, hybrids backcross to the northern but not the southern lineage, and nearly all hybrid individuals have northern lineage maternally inherited mitochondrial DNA (mtDNA), indicating some degree of reproductive isolation (Dong et al. 2019).

The lineages of *C. decresii* differ in male throat coloration, which they display prominently during both contests and courtship (Gibbons 1979; Stuart-Fox and Johnston 2005; Osborne et al. 2012; Ramos and Peters 2016). Northern lineage males are color polymorphic, with orange, yellow, orange–yellow (an orange central patch surrounded by yellow), and gray throat morphs co-occurring within populations (Teasdale et al. 2013; McLean et al. 2014b). Conversely, southern lineage males have blue throats with an ultraviolet (UV) reflectance peak (McLean et al. 2014b). Northern lineage color morphs also exhibit correlated behavioral strategies; gray males are the most cautious, while orange males are the most bold and the most aggressive toward territory intruders (Yewers et al. 2016). Females are cryptically colored and similar in appearance between lineages (Figure 1; McLean et al. 2013). The species occupies open, rocky habitats and males defend territories using elaborate behavioral displays from elevated rock perches, sometimes engaging in physical contests (Gibbons 1979; Stuart-Fox and Johnston 2005; Osborne et al. 2012; Ramos and Peters 2016). Males are polygynous with territories encompassing the home ranges of one or more females (Yewers et al. 2018); thus, a male's ability to defend a territory likely influences reproductive success. Parental care is absent and multiple paternity within clutches is rare (4%), though this does not preclude female multiple mating within or between clutches due to the possibility of sperm storage and sperm competition (Hacking et al. 2017). Consequently, the biology as well as the mating system of *C. decresii* suggests strong intra-sexual selection.

In this study, we staged encounters between captive lizards from populations representing the closest genetically pure populations on either side of the contact zone. This enabled us to assess the extent to which divergent mate preferences have evolved in the 2 lineages, independently of reinforcement subsequent to secondary contact. Doing so is essential to assess the role of sexual selection in the speciation process. We predicted that lizards would display a preference for mates from their own lineage. If this is due to female preference, females should perform more courtship and/or less rejection behavior during encounters with males from the same lineage. Similarly, if male preference plays a role then males should be more likely to court and/or attempt copulation with females from their own lineage as opposed to the alternate lineage. Additionally, we predicted that northern and southern males would differ in their aggressive behavior, and that this behavior may be dependent on the throat color morph of their opponent.

Materials and Methods

Study species and husbandry

We used 90 adult lizards (>65 mm snout–vent length; SVL) comprising 21 male and 24 female northern lineage *C. decresii* from Caroona Creek Conservation Park, South Australia (−33.4114°S, 139.0945°E), and 21 male and 24 female southern lineage *C. decresii* from private properties around Palmer, South Australia (−34.8223°S, 139.1621°E). Lizards were collected in September in 2015 and 2016, and subsequently kept in captivity at The University of Melbourne, Victoria, Australia, where they were housed individually in 55 × 34 × 38 cm (length × width × height) opaque plastic enclosures containing a layer of sand and a crevice between 2 ceramic tiles for shelter. Housing was maintained at temperatures and lighting cycles that mimicked natural seasonal variation, with UV lights (ZooMed T8 ReptiSun® 10.0 UVB) above each enclosure (30 cm), emitting both UVA and UVB radiation. A heat lamp was provided to generate a thermal gradient and allow the lizards to attain their preferred body temperatures (approx. 36°C; Gibbons 1977; S. Walker, unpublished data). Lizards were misted with water for hydration and fed live crickets dusted with multi-vitamins 3 times per week. All behavioral trials were conducted during the breeding seasons (August–December; Gibbons 1977) in 2016 and 2017. Research methods used in this study were reviewed and approved by the Animal Ethics Committee of The University of Melbourne (1413220.3) and the South Australian Wildlife Ethics Committee (25/2015).

Female–male behavioral trials

Females are receptive to mating approximately 2–3 weeks after emergence from hibernation, and after laying their first or second clutch. We conducted mate preference trials during these known receptive periods, when females were in good body condition (average

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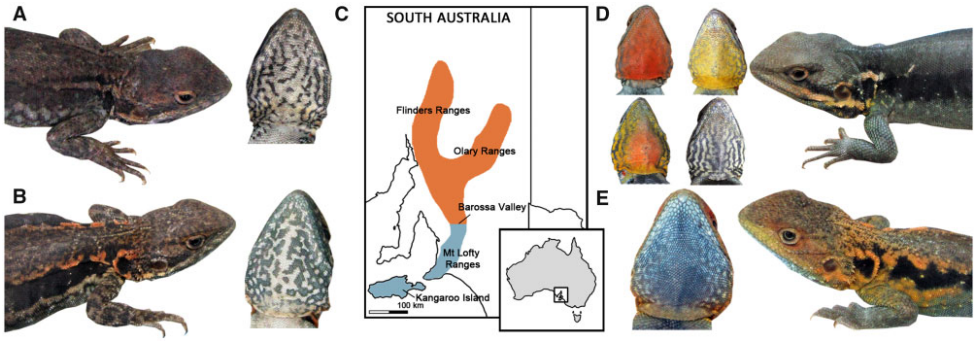


Figure 1. Phenotypic variation between the northern and southern lineages of *C. decresii*. Parts (**A**) and (**B**) show northern and southern lineage females, respectively, which are similar in appearance. (**C**) Distribution of the 2 lineages (northern = orange shading; southern = blue shading). Parts (**D**) and (**E**) show northern and southern lineage males, respectively. Northern males are polymorphic for throat coloration.

mass of 16.7 ± 2.9 g), though receptivity cannot be determined with certainty *a priori*. Each female was paired with both a southern and a northern lineage male, with half of the females paired with a southern male first and the other half with a northern male first. Females were placed into the first male's enclosure for a period of 24 h, and then into the second male's enclosure for the subsequent 24 h. Both encounters were monitored and recorded using a Swann DVR8-1525 8 channel 960H digital video recorder with a PRO-615 camera attached. We conducted a total of 147 trials, with individual females paired with one southern and one northern male per reproductive cycle, in up to 2 reproductive cycles (average of 3.34 trials, with a range of 2–4 trials, per female).

Videos were analyzed using Behavioral Observation Research Interactive Software (BORIS) version 4.1.5 (Friard and Gamba 2016) and both female and male behavior was scored. For females, we recorded the number of head-bobs (pronounced nodding movement of the head), and combined the number of aggressive behaviors (biting and chasing) and times the female fled from the male as a measure of "rejection." For males, we also recorded the number of head-bobs (courtship behavior) as well as the number of attempts to copulate, and whether or not copulation was successful. We did not analyze the number of successful copulations as copulation was observed in only 7 of the 147 trials (although more may have taken place under the tile). Lizards were not paired for long enough to ensure mating; rather, we were interested in behavior during initial contact as an indicator of mate preference.

We tested whether female lineage, male lineage, or their interaction predicted: 1) number of copulation attempts, 2) number of male head-bobs, 3) number of female head-bobs, and 4) number of female rejection behaviors using generalized linear mixed models (lme4 package, R; Bates et al. 2015). Female ID, male ID, and pairing number (female's first or second trial) were included as random factors in all models to account for repeated use of individuals, and response variables were log transformed to meet model assumptions of normality. We performed pairwise comparisons by calculating least squares means and confidence intervals using the Satterthwaite's approximation for degrees of freedom (lmerTest package, R; Kuznetsova et al. 2017).

Male–male behavioral trials

A previous study investigating aggression levels among morphs of the northern lineage found that orange-throated males were significantly more aggressive toward territory intruders than yellow, orange–yellow, or gray-throated males (Yewers et al. 2016). Therefore, we categorized males into 3 behavioral groups based on lineage and throat color morph: 1) southern, 2) northern high aggression (orange), or 3) northern low aggression (yellow, orange–yellow, gray). We designed trials such that each focal male was matched with 3 others, representing each of the behavioral groups, in random order. Pairs were size-matched to minimize the effect of body size on contest outcome, with an average difference of 1.59 ± 1.16 mm SVL between competing males.

Contest trials were conducted in a neutral 120 × 30 × 60 cm (length × width × height) enclosure (i.e., not the home enclosure of either male). An opaque divider initially separated the enclosure into 2 equally sized holding areas, each containing a layer of sand, ceramic tile, and heat lamp. Just prior to the trial, males were weighed to obtain a measure of body condition as the residuals of a linear model of mass and SVL. The designated "focal" and "opponent" males were then placed into the separate holding areas and allowed to acclimatize for 48 h to establish residency (Umbers et al. 2012). At the commencement of the trial, the divider was removed and the interaction was recorded from 2 different angles using Panasonic HC-V770M video cameras. Trials were conducted for a maximum of 35 min and monitored to ensure there was no risk of injury to animals (as required under the Animal Ethics permit). Consequently, we did not record contest outcome (i.e., winner, loser) as some trials were stopped before a winner was established. To minimize stress and the potential influence of previous contest outcomes, males were not used in a subsequent trial for at least 48 h. We conducted a total of 120 trials (involving 42 males), 26 of which were excluded due to no interaction, resulting in 94 trials which were used in the statistical analysis.

We scored focal male behavior from the video footage using BORIS. *Ctenophorus decresii* males perform energetic displays during territory defense prior to engaging in physical aggression (Gibbons 1979). Therefore, we recorded the number of head-bobs, tail flicks, and push-ups performed by the focal male as a measure of "display behavior," and combined the duration of chasing and wrestling (involving biting) as a measure of "physical aggression." We also recorded the time between the start of the trial and the focal male's emergence from beneath the tile ("latency"), as this is an indicator of individual boldness. Display behavior and physical

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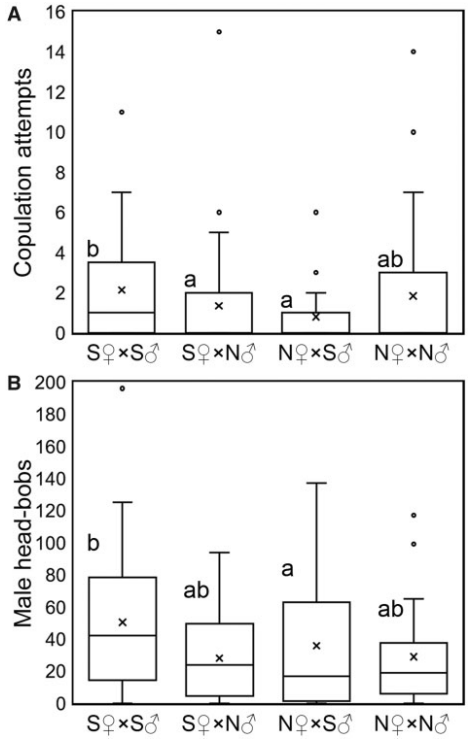


Figure 2. The total number of (**A**) copulation attempts and (**B**) male head-bobs (courtship) performed during mate preference trials between females and males of the southern (S) and northern (N) lineages of *C. decresii*. Letters indicate statistically significant differences between lineage pairings.

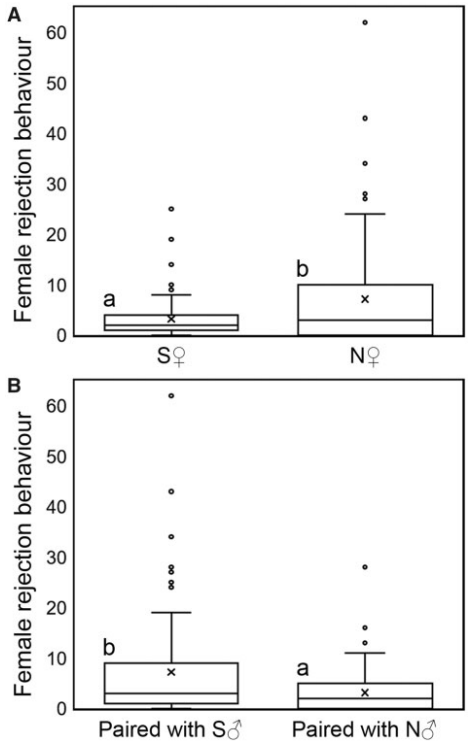


Figure 3. Comparison of (**A**) the total number of rejection behaviors (biting, chasing, and fleeing) performed by southern (S) and northern (N) females and (**B**) the number of rejection behaviors performed by females (regardless of lineage) when paired with southern and northern males. Letters indicate statistically significant differences between lineages.

aggression were divided by the total trial duration (minus latency) to account for differences in trial lengths.

We tested whether behavioral group or body condition predicted: 1) focal male latency to emerge, 2) focal male display behavior, and 3) focal male physical aggression using generalized linear mixed models. We included focal male behavioral group, opponent male behavioral group and their interaction, as well as focal male body condition and opponent male body condition as predictor variables in the models. Additionally, focal male ID and focal male trial number were included as random factors in all models to account for repeated use of individuals. For models 2 (display behavior) and 3 (physical aggression), the response variables were log transformed to meet model assumptions of normality, and we performed *post hoc* pairwise comparisons as detailed above.

Results

Female–male behavioral trials

The interaction between female and male lineage influenced the number of copulation attempts ($F_{1,103.2} = 6.53$, $P = 0.012$) and male head-bobs (courtship; $F_{1,91.2} = 6.61$, $P = 0.012$) during trials (Table 1). Males attempted copulation in 68 of the 147 trials and there were more copulation attempts between southern males paired with southern females than southern males paired with northern females ($t_{107.8} = 2.46$, $P = 0.015$) or northern males paired with southern females ($t_{63.9} = 2.00$, $P = 0.050$; Figure 2 and Supplementary Table S1). Similarly, southern males courted southern females (with head-bobs) more than they courted northern females ($t_{91.3} = 3.21$, $P = 0.018$; Figure 2 and Supplementary Table S2). Lineage did not influence female head-bobs; however, both female and male lineage affected female rejection behavior (Table 1). Specifically, northern females performed more rejection behaviors than southern females ($F_{1,40.2} = 4.64$, $P = 0.037$) and southern males were rejected more overall (i.e., by females of both lineages) than northern males ($F_{1,38.9} = 10.06$, $P = 0.003$; Figure 3).

Male–male behavioral trials

Focal males displayed (head-bobs, tail flicks, and push-ups) in 73 out of 94 contest trials, and 70 trials involved physical aggression (chasing and wrestling). Focal male ID (a random factor) explained a large proportion (57.07%) of the variance in the time taken for the focal male to emerge from beneath the tile (latency). Taking this into account, latency was affected by both the focal male's

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Table 1. Results of generalized linear mixed models investigating whether female and male lineage (southern and northern) predicts behavior during mate preference trials

Behavior	Variable	R^2m	R^2c	Estimate (95% CI)	F_{df}	P
(1) Copulation attempts	Female lineage	0.05	0.12	−0.067 (−0.198, 0.064)	$1.05_{1,41.5}$	0.311
	Male lineage			−0.018 (−0.139, 0.102)	$0.08_{1,30.5}$	0.780
	Female lineage × male lineage			0.149 (0.033, 0.264)	$6.53_{1,103.2}$	*0.012*
(2) Male head-bobs	Female lineage	0.07	0.22	−0.272 (−0.540, 0.006)	$4.12_{1,31.9}$	0.051
	Male lineage			−0.030 (−0.273, 0.224)	$0.06_{1,28.8}$	0. 805
	Female lineage × male lineage			0.293 (0.066, 0.519)	$6.61_{1,91.2}$	*0.012*
(3) Female head-bobs	Female lineage	0.04	0.27	0.189 (−0.033, 0.412)	$2.91_{1,41.1}$	0.096
	Male lineage			−0.087 (−0.240, 0.067)	$1.26_{1,101.3}$	0.265
	Female lineage × male lineage			0.018 (−0.135, 0.171)	$0.05_{1,100.6}$	0.817
(4) Female rejection	Female lineage	0.11	0.22	0.178 (0.009, 0.341)	$4.64_{1,40.3}$	*0.037*
	Male lineage			−0.279 (−0.457, −0.103)	$10.1_{1,38.9}$	*0.003*
	Female lineage × male lineage			−0.074 (−0.227, 0.079)	$0.92_{1,103.9}$	0.340

Statistically significant relationships are italicized. CI, confidence interval; R^2m, marginal R^2; R^2c, conditional R^2; *df*, degrees of freedom.

behavioral category ($F_{2,41.5} = 4.35$, $P = 0.019$) and opponent male's behavioral category ($F_{2,58.1} = 5.45$, $P = 0.007$) but neither male's body condition (Table 2). The relationships were such that northern low aggression males (yellow, orange–yellow, and gray morphs) were slower to emerge than southern males ($t_{39.5} = 2.92$, $P = 0.006$), and the focal male emerged more quickly for a southern ($t_{59.1} = 3.05$, $P = 0.003$) or northern low aggression ($t_{56.9} = 2.26$, $P = 0.028$; Supplementary Table S3) opponent compared with a northern high aggression (orange morph) opponent (Figure 4 and Supplementary Table S4). Neither male behavioral category nor body condition affected display behavior or physical aggression during trials (Table 2).

Discussion

Speciation via sexual selection requires sexual signals and mating preferences to diverge together, resulting in assortative mating upon secondary contact. Males of the northern and southern lineages of *C. decresii* differ markedly in male throat coloration, a signal used in both intra- and inter-sexual interactions. Mating trials between the lineages showed that southern males display a preference for females from their own lineage. Southern males courted and attempted copulation with southern females more than northern females. By contrast, northern males did not court or attempt copulation with northern females more than with southern females. Additionally, northern females displayed more rejection behaviors than southern females and southern males were rejected more (regardless of female lineage) than northern males. These data suggest that both male and female mate preferences may act as behavioral barriers to gene flow upon secondary contact. However, we found no evidence for behavioral barriers to mating between northern males and southern females, suggesting that the evolution of premating isolation prior to secondary contact is incomplete and asymmetrical.

Divergent sexual selection can theoretically drive speciation, even in the absence of reinforcement (Lorch et al. 2003; Reinhold 2004; van Doorn et al. 2009). This is most likely when sexual signals are condition-dependent or directly under natural selection because selection against locally maladapted offspring should generate linkage disequilibrium between trait and preference, ultimately leading to assortative mating (Maan and Seehausen 2011). Male throat coloration in *C. decresii* is locally adapted to increase conspicuousness against the color of the dominant lichens on rocks in the

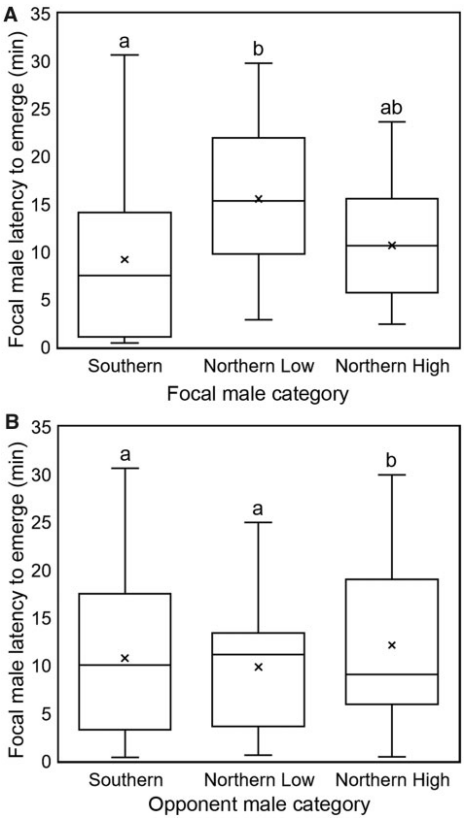


Figure 4. Time taken (in minutes) for (**A**) southern (blue), northern low aggression (yellow, orange–yellow, gray morphs), and northern high aggression (orange morph) focal males to emerge from beneath the tile and (**B**) focal males to emerge depending on the behavioral category of the opponent male. Lowercase letters indicate significant differences between behavioral categories. Note that focal male ID explains a large proportion of the variance in latency to emerge.

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Table 2. Results of generalized linear mixed models investigating whether male behavioral category or condition predict behavior during male–male contests

Behavior	Variable	R^2m	R^2c	SS	F_{df}	P
(1) Latency	FM behavioral category	0.22	0.73	166.8	$4.35_{2,41.5}$	*0.019*
	OM behavioral category			209.0	$5.45_{2,58.1}$	*0.007*
	FM condition			73.4	$3.83_{1,91.2}$	0.053
	OM condition			63.4	$3.31_{1,75.7}$	0.073
	FM behavioral category×OM behavioral category			73.0	$0.95_{4,57.2}$	0.441
(2) Display behavior	FM behavioral category	0.14	0.33	7.7×10^{-5}	$0.84_{2,32.5}$	0.441
	OM behavioral category			6.1×10^{-5}	$0.66_{2,58.5}$	0.519
	FM condition			6.1×10^{-6}	$0.13_{1,65.6}$	0.717
	OM condition			5.0×10^{-5}	$1.09_{1,92.6}$	0.300
	FM behavioral category×OM behavioral category			4.4×10^{-4}	$2.39_{4,56.2}$	0.062
(3) Physical aggression	FM behavioral category	0.08	0.09	0.061	$1.35_{2,93.5}$	0.264
	OM behavioral category			0.041	$0.90_{2,93.6}$	0.410
	FM condition			0.030	$1.33_{1,92.6}$	0.251
	OM condition			7.2×10^{-6}	$3.0\times10^{-4}{}_{1,94.0}$	0.986
	FM behavioral category×OM behavioral category			0.011	$0.12_{4,92.8}$	0.973

Behavioral categories are: "southern," "northern low aggression" (yellow, orange–yellow, and gray morphs) and "northern high aggression" (orange morph). Statistically significant relationships are italicized. SS, type III sum of squares; R^2m, marginal R^2; R^2c, conditional R^2; df, degrees of freedom; FM, focal male; OM, opponent male.

habitats of the 2 lineages (McLean et al. 2014a). Southern blue throats are more conspicuous against the orange lichen found extensively on rocks in the south, while northern orange and yellow throats are more conspicuous against the gray–green lichen that dominates in the north (McLean et al. 2014a). Despite local adaptation of the color signal, we found little evidence for a strong link between signal and preference: southern females did not discriminate between northern and southern males, and northern males did not show a preference for northern females. Thus, the strength of divergent ecological selection on sexual signals in this system appears insufficient to generate strong linkage between sexual trait and preference, and therefore assortative mating upon secondary contact.

Mate discrimination or choosiness is expected to be stronger in the sex that invests more into reproduction per offspring, typically females (Anderson 1994). Thus, premating isolation is generally assumed to be driven by female preferences. In lizards, which mostly lack parental care, females invest more per offspring into reproduction; yet, evidence for female mate choice is scarce (López et al. 2003; Olsson et al. 2003; Martín and López 2006). This is because males largely control female access to mates, either by dominating territories encompassing the home range of one or more females, or by mate guarding (Olsson and Madsen 1995). We do find some evidence suggesting a role for female mate preference in *C. decresii*. Like most lizards, female *C. decresii* mate multiply when receptive, but this does not mean they mate indiscriminately (Elgar et al. 2013), and they may exhibit mate preferences through rejection of non-preferred mates. Our results showed that northern females exhibited more rejection behavior, and that southern males were rejected more overall, which could have contributed to the lower number of copulation attempts observed between this combination.

Male–male competition is unlikely to drive speciation on its own, but may contribute to reproductive isolation in combination with mate preferences or genetic incompatibilities. For example, in European wall lizards *Podarcis muralis*, asymmetric introgression occurs due to strong differences between lineages in male competitive ability and male mate preferences, which are both linked to body size (While et al. 2015; Heathcote et al. 2016). Correlated evolution of body size and sexual signals is a common pattern (Young et al. 1994; Wirtz 1999; Hagman and Forsman 2003; McGlothlin et al. 2005; reviewed in Bonduriansky 2007). In *C. decresii*, males of the 2 lineages differ very slightly in body size and head shape (McLean et al. 2013), but did not differ in competitive ability in terms of aggression during staged contests. Thus, we find no evidence that male–male competition contributes to incipient speciation in *C. decresii*, despite the importance of intra-sexual selection in this species, and its widely acknowledged importance in lizards more generally.

Although lineages did not differ in aggression, there were differences in boldness between lineages and morphs, consistent with previously described behavioral differences between the northern throat color morphs (Yewers et al. 2016). Southern males were bolder (faster to emerge from their shelter) than northern low aggression (yellow, orange–yellow, and gray) males and equally bold as northern high aggression (orange) males. Overall, focal males were least bold when faced with a northern high aggression (orange) opponent. In other species, higher boldness has been associated with greater reproductive success, foraging ability, dispersal distance, and dominance, but may reduce long-term survival (Réale et al. 2007; Ariyomo and Watt 2012). The relationship between boldness and reproductive success in reptiles is relatively unexplored but it has been suggested that boldness may be associated with the ability to defend territories, but is not necessarily correlated with social dominance and aggression (Taylor and Lattanzio 2016).

In summary, we have shown that both male and female mate preferences may potentially influence the nature and extent of reproductive barriers upon secondary contact between lineages of *C. decresii*. We found little evidence of lineage differences in male competitive ability, despite the importance of male–male competition for male reproductive success in *C. decresii* and lizards in general. Furthermore, the marked divergence in male throat coloration is only weakly correlated with divergence in mate preferences, which is also asymmetric. This suggests that divergent sexual selection alone is unlikely to be the primary driver of incipient speciation between the 2 lineages. Instead, genetic data (asymmetric introgression and northern mtDNA in hybrids) suggest a role for genetic

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incompatibilities that have arisen during the independent evolution of the lineages (Dong et al. 2019). More broadly, our data are consistent with the emerging view that sexual selection alone rarely drives speciation to completion (Ritchie 2007; Maan and Seehausen 2011; Scordato et al. 2014; Lackey and Boughman 2017; Servedio and Boughman 2017).

Data Accessibility

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.6m905qfws

Author Contributions

C.A.M., R.A.B., and D.S.-F. designed the study. C.A.M., R.A.B., C.M.D., and K.J.R. contributed to the animal collection and maintenance. R.A.B. conducted the experiments and extracted the behavioral data. C.A.M. performed the statistical analyses. C.A.M., D.S.-F., and C.M.D. drafted the manuscript. All authors contributed to the interpretation and revision of the manuscript.

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Supplementary Material

Supplementary material can be found at https://academic.oup.com/cz.

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